Charles University

Faculty of Science

Study programme: Biology

Branch of study: Animal Physiology

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Localization of cytosolic isoforms of creatine kinase and hexokinase in hypertrophied heart

Lokalizace cytosolických izoforem kreatin kinázy a hexokinázy v hypetrofovaném srdci

Diploma thesis

Supervisor: RNDr. Jitka Žurmanová, Ph.D.

Prague 2017

Declaration

I hereby declare that I completed this Diploma thesis independently, except where explicitly indicated otherwise. It documents my own work, carried out under the supervision of RNDr. Jitka Žurmanová, PhD. Throughout, I have properly acknowledged and cited all sources used. Neither this thesis nor its substantial part has been submitted to obtain this or other academic degree.

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Acknowledgement

I am heartily thankful to my dear supervisor RNDr. Jitka Žurmanová, PhD. for her understanding, guidance and encouragement during my study. I would also like to thank to my expert consultant RNDr. Barbara Elsnicová, PhD. for sharing of her endless experience in microscopic field. I would like to express my most sincere thanks to all dear colleagues who contributed to this work and were always willing to help and created a pleasant work environment: Daniela Hornikova, Pavel Vebr, Aneta Marvanová, David Kolář, Veronika Tibenská, Jana Kohútová and Iveta Brabcová. Last but not least, I would like to thank my family and friends for their love, patience, understanding and constant support.

Abstrakt

Hypertrofie srdce je úzce spjatá s metabolickými adaptacemi a buněčnou remodelací. Významný a dynamický systém podílející se na udržení energetické homeostázy v rozvoji hypertrofie je kreatin-kinázový systém (CK). Mikrokompartmentalizace CK izoforem udržuje tok ATP mezi místy jeho spotřeby a produkce a zabezpečuje efektivitu CK systému. Je známo, že v průběhu hypertrofie dochází k změnám v expresi a aktivitě jednotlivých izoforem CK – tato studie rozšiřuje toto poznání o kvantifikaci změn v asociaci cytosolických izoforem CK v oblasti sarkomery. Druhý významný systém, který udržuje homeostázu v přetíženém srdci je tvořen izoformami hexokinázy (HK), je též lokalizován v cytosolu a v oblasti mitochondrií. Za fyziologických podmínek je izoforma HK1 asociována s mitochondriemi, kde udržuje mitochondriální membránový potenciál, zatímco HK2 je lokalizována převážně v cytosolu. V zátěžových podmínkách dochází k translokaci HK2, což zvyšuje přímou dodávku ADP ke komplexu V respiračního řetězce a snižuje pravděpodobnost aktivace apoptózy. V rámci druhého cíle této práce jsme analyzovali asociaci jednotlivých izoforem hexokinázy (HK) s mitochondriemi. Třetím cílem práce bylo charakterizovat změny v asociaci CK s M liniemi po ischemii a reperfúzi u potkanů adaptovaných na kardioprotektivní režim intermitentní hypobarické hypoxie (IHH), který rozvíjí hypertrofii v obou komorách.

Klíčové slova: srdce, hypertrofie, kreatin kináza, hexokináza.

Abstract

Hypertrophy of the heart is tightly bound to the metabolic adaptations and a cellular remodeling. An important and dynamic system contributing to the maintenance of energy homeostasis is the creatine kinase system (CK). The microcompartmentalization of CK isoforms maintains the flux of ATP between energy production and consumption sites and ensures the effectiveness of the CK system. Changes in expression and activity of CK isoforms during hypertrophy are already well described – to extend this knowledge, this thesis quantified changes in association of cytosolic CK isoforms and sarcomeres. Another essential system, maintaining homeostasis in overloaded heart is composed of the hexokinase (HK) isoforms, located also in cytosol and in mitochondrial compartment. HK1 is associated with mitochondria under physiological conditions, maintaining mitochondrial membrane potential, while HK2 is located mainly in the cytosol. Under stress conditions translocation of HK2 into mitochondrial membrane occurs, which increases the direct supply of ADP to complex V of the respiratory chain and decreases the probability of apoptosis activation. We analyzed association of individual HK isoforms with mitochondria within the second aim of this thesis. Third aim of the thesis was to characterize changes in the CK and M line association after ischemia and reperfusion in rats adapted to cardioprotective regimen of intermittent hypobaric hypoxia (IHH), which develops hypertrophy in both ventricles.

Keywords: heart, hypertrophy, creatine kinase, hexokinase.

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List of abbreviations

ADP	adenosine diphosphate
AMPK	AMP-activated protein kinase
ANF	atrial natriuretic factor
ANT	adenine nucleotide transporter
ATP	adenosine triphosphate
Bax	Bcl-2 associated X protein
BNP	brain natriuretic factor
BW	body weight
β-AR	β-adrenergic receptors
CaMKII	signaling and calmodulin-dependent protein kinase II
CK	creatine kinase
CKB	creatine kinase brain isoform
CKBB	creatine kinase brain homodimer
CKM	creatine kinase muscle isoform
CKMB	creatine kinase muscle/brain heterodimer
CKMM	creatine kinase muscle homodimer
CKs	sarcomeric creatine kinase
Cr	creatine
DAPI	4’,6-diaminidino-2-phenylindole
ECM	extracellular matrix
ERK1/2	extracellular signal-regulated kinase 1/2
FAK	focal adhesion kinase
FOX	Forkhead box
G-6-P	glucose-6-phosphate
GLUT1	glucose transporter 1
GLUT4	glucose transporter 4
GSK-3β	glycogen synthase kinase 3β
HF	heart failure

HIF-1	hypoxia-inducible factor 1
HK	hexokinase
IGF-1R	insulin-like growth factor 1 receptor
ILK	integrin-linked kinase
IR	ischemia - reperfusion
LV	left ventricle
MAP3K	mitogen activated protein kinase kinase kinase
MCIP1	modulatory calcineurin-interacting protein-1
mTOR	mechanistic target of rapamycin
MyHCα	myosin heavy chain α
MyHCβ	myosin heavy chain β
NFAT	nuclear factor of activated T-cells
NMR	nuclear magnetic resonance
NRF1/2	nuclear respiratory factor 1/2
PCG-1α	peroxisome proliferator-activated receptor gamma co-activator
PCr	phosphocreatine
PCR	polymerase chain reaction
PI3K	phosphatidylinositol-4,5-bisphosphate 3-kinase
PKC	protein kinase C
PLC	phospholipase C
PTP	mitochondrial permeability transition pore
ROS	reactive oxygen species
RV	right ventricle
SERCA	sarco/endoplasmic reticulum Ca^{2+} ATPase
SHR	spontaneous hypertensive rats
T3	triiodothyronine
T4	thyroxin
TH-R	thyroid hormones receptors
VDAC	voltage-dependent anion channel
WKY	Wistar Kyoto rats

1. Introduction

Systemic hypertension is a serious civilization disease, which leads to left ventricular hypertrophy and heart failure (HF). Despite the well described mechanisms associated with hypertrophy, many uncertainties remain in the transition period between reversible compensation phase and decompensation phase characterized by irreversible changes. The changes in substrate channeling mechanism between myofibrillar creatine kinase (CK) and myosin ATPase might be closely related to this transition and possess high impact on heart contractility. Alterations of the expression of CK isoforms in failing myocardium are known, but their localization in relation to sarcomeres, which is critical for the contractile function, has not been studied in the course of hypertrophy yet. Hexokinase as a main regulatory enzyme of the glucose metabolism is closely related to the metabolic changes happening during cardiac hypertrophy. Second aim of this thesis is to describe changes in the colocalization of the hexokinase isoforms with mitochondria which might affect functional coupling of glycolysis with oxidative phosphorylation. Aside from expression, binding of the hexokinase to the outer mitochondrial membrane has a potent oxidative phosphorylation stimulating and antiapoptotic effects, therefore we assume that dynamics in hexokinase – mitochondria colocalization in course of the cardiac hypertrophy might reveal cardioprotective effect.

Lastly, this thesis examines the shift in location of cytosolic CK isoforms within the sarcomere after ischemia and ischemia - reperfusion (IR) in rats adapted to cardioprotective regimen of intermittent hypobaric hypoxia in comparison with animals kept in the normal barometric pressure environment. Myocardium in hypoxic conditions adapts through various metabolic mechanisms, involving CK system.

Hence this thesis targets possible role of different dynamics of CK in sarcomeric and HK in mitochondrial compartment under pathological conditions of heart.

2. The literature Review

2.1. Heart hypertrophy

Oxidative heart tissue has high energy demand, fluctuating in accordance to activity of the organism. Chronic pressure or volume heart overload leads to a hypertrophic growth, which compensates higher output demand on left or right ventricle. Under physiological conditions, mammalian cardiomyocytes exit the cell cycle soon postnatally and hypertrophic growth becomes major postnatal mechanism of the myocardial maturation. Hypertrophic growth is accompanied by angiogenesis, which maintains oxygen and substrate delivery and energy homeostasis of myocardium with rising output demand (Hudlicka & Brown, 1996; Shiojima et al., 2005)

Major mechanism of hypertrophic growth lies in the increased protein synthesis and in the alterations in gene expression. Hypertrophic signaling is mediated through β-adrenergic receptors (β-AR), insulin-like growth factor 1 receptor (IGF-1R), mechanoreceptors and thyroid hormones receptors (TH-R). These receptors mediate the most important hypertrophic signaling pathways of IP3/Akt kinase, renin-angiotensin-aldosterone pathway and thyroxin (T4) and triiodothyronine (T3) signaling, respectively. All of the main signaling pathways upregulating protein synthesis converge either to mitogen-activated protein kinase kinase kinase (MAP3K) and extracellular signal-regulated kinase 1/2 (ERK1/2) (mechanic stress stimulus, angiotensin) or to intracellular receptors translocated to the nucleus upon activation. Especially important is Akt signaling in hypertrophy, with Akt stimulating glucose metabolism and upregulating expression of proteins of Forkhead box (FOX) family involved in cell growth (for review Sussman et al., 2011). Shiojima et al. (2005) showed that active Akt can lead to both physiological and pathological hypertrophy and therefore Akt might play an important role in the transition phase between them. Akt, activated by insulin receptor and mechanoreceptor cascades, is also involved in upregulation of protein synthesis through direct activation of mechanistic target of rapamycin (mTOR), considered as a main hypertrophy mediator of Akt signaling pathway and upregulation of protein translation through inhibition of glycogen synthase kinase 3β (GSK-3β). Structural sarcomeric proteins upregulated by the hypertrophic

signaling are myosin heavy chains β (MyHCβ) with slow ATPase activity, α actin and EH-myomesin, splice isoform of myomesin 1 (Nakao et al., 1997; Schoenauer et al., 2011).

During hypertrophic growth, shape and/or volume of the ventricle is changed, depending on the type of the stress imposed on heart and hemodynamic factors that lead to the increased work load. If the myocardium is chronically forced to exert higher than physiological contraction force, as in the case of aortic stenosis or hypertension, the stimulus leading to hypertrophic growth is pressure overload. Pressure overload leads to the concentric hypertrophy, characterized by increased wall thickness. Wall thickening is achieved by creating new sarcomeres parallel to existing sarcomeres and remodeling of extracellular matrix (ECM). In physiological phase of concentric hypertrophy, the thickness of myocardial wall is proportionate to force needed to overcome increased pressure and stress is distributed evenly across the myocardial wall. Excessive concentric hypertrophy leads to the decrease of the cavity volume and decreased flexibility of myocardium which leads to the impairment of diastolic function.

Volume overload occurs under certain condition when the myocardium is chronically forced to work with higher than physiological volume of blood. Then the volume overload can result in the eccentric hypertrophy, characteristic by enlargement of the ventricular cavity, allowing heart to accept higher volume of blood. Newly synthesized and formed sarcomeres in eccentric hypertrophy are placed in series with existing sarcomeres. Ventricle is enlarged laterally inside thoracic cavity and wall thickness might decrease during eccentric hypertrophy. Some of the pathologies causing eccentric hypertrophy are mitral and aortic insufficiency (Grossman et al., 1975; Shimizu & Minamino, 2016).

Nature of the stress is the main factor determining phenotype of cardiac hypertrophy. Because hypertrophy can progress from compensatory to the pathological phase, duration of stimulus is another factor determining nature of hypertrophic growth. In general, signaling that leads to physiological hypertrophy is dysregulated by chronic or overloading stimulation and myocytes switch from compensatory to fibrotic remodeling pattern (Shiojima et al., 2005).

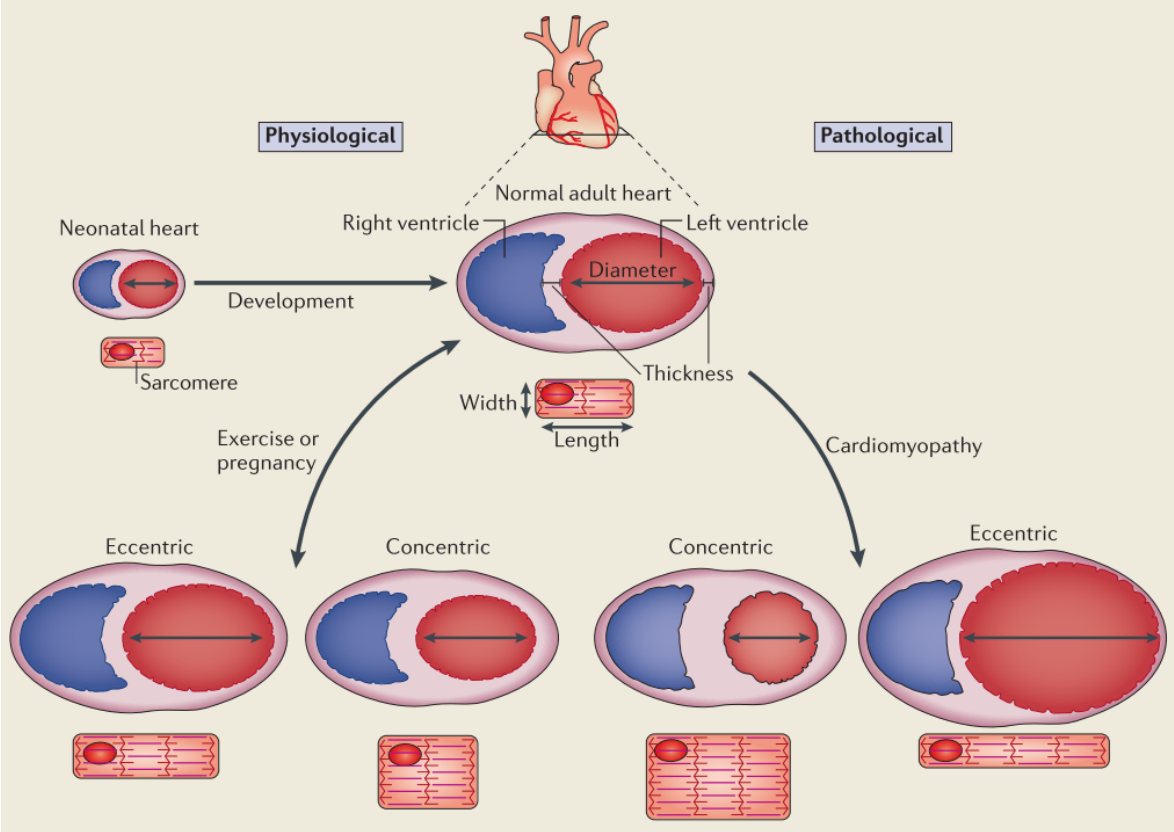

Fig. 1: *Visualization of a different cardiac phenotypes and geometry of the human heart. Schematic image of transverse section of the healthy heart with physiological ratio of wall thickness to ventricle diameter is on the top of the figure with normal cardiomyocyte underneath. Differences between physiological hypertrophy, induced by pregnancy or exercise and pathological hypertrophy induced by cardiomyopathy are visible at the bottom of the figure. Eccentric hypertrophy, induced by volume overload, develops by creating new sarcomeres in series with existing sarcomeres. Eccentric remodeling leads to the ventricle dilatation and even to the dilated cardiomyopathy in pathological phase. Concentric hypertrophy, induced by pressure overload, develops by creating new sarcomeres in parallel with existing sarcomeres. Concentric hypertrophy leads to the ventricle wall thickening, decreased compliance and even to the diastolic dysfunction in case of pathological hypertrophy (Maillet et al., 2013).*

2.2. Physiological hypertrophy

Plasticity of cardiac tissue enables the heart to adapt to increased demand and thus does not need to be detrimental, at least not in initial phase. In general, if hypertrophic growth as a response to physiological stress does not differ from enlargement of myocardium under physiological conditions (as in pregnancy or trained athletes), the heart maintains its normal function and the remodeling is reversible. Then these adaptive changes are generally accepted as physiological hypertrophy, or physiological phase of hypertrophy. Fibrosis does not develop in this compensatory phase of hypertrophy due to the maintained normal collagen isoforms ratio and preserved collagen network organization (Maillet et al., 2013).

One of the important markers of cardiac function, the wall thickness to ventricle diameter ratio, is also maintained in physiological range. Therefore, heart in physiological phase of hypertrophy is able to increase its work output and keep its cardiac function and stiffness in physiological range. Mitochondrial biogenesis is activated by peroxisome proliferator-activated receptor gamma co-activator (PCG-1α), which increases expression of nuclear respiratory factor 1 (NRF1) and nuclear respiratory factor 2 (NRF2) genes. Increased level of PCG-1α was observed after physical training, when PCG-1α activated mitochondrial biogenesis and support oxidative metabolism (O'Neill et al., 2007; Watson et al., 2007).

Critical mechanism maintaining proper nourishment of the cardiomyocytes during growth of the cardiac tissue is proportional angiogenesis of the coronary arteries, supplying oxygen and nutrients to the ventricles. Cardiac function is strictly dependent on the coordination between angiogenesis and tissue growth. This balance is retained in the physiological hypertrophy and progressive dysregulation follows in development of the pathological hypertrophy. The prominent role of Akt kinase had been shown in the regulation of this coordination. However dysregulation of the angiogenesis and growth, mediated by excessive activity of Akt, emphasize important role of Akt in the shift between physiological and pathological hypertrophy (Shiojima et al., 2005).

Increased energetic substrates utilization is required to match both higher workload and protein synthesis. Rat hearts with developed hypertrophy based on aerobic training showed 17% increase in oxidative capacity accompanied by increase of PPAR-α, medium-chain acyl-CoA dehydrogenase (MCAD), carnitine palmitoyltransferase I (CPTI) expression and fatty acid utilization in trained rats group exclusively (+58% PPAR-α, +66% MCAD, +59% CPTI). However, increased oxidative capacity was not observed in 15-week old spontaneously hypertensive rats (SHR) (10% cardiac hypertrophy) and 25-week old SHR group (35% cardiac hypertrophy) (Rimbaud et al., 2009).

Physiological hypertrophy in trained rats induced alterations in glucose utilization. Glucose transporter of the plasma membrane GLUT4 was upregulated by 88%. On the other hand, pyruvate dehydrogenase kinase 4 (PDK4), phosphofructokinase and lactate dehydrogenase expression was not changed by exercise training, suggesting that physiological hypertrophy increases glucose turnover by oxidation but not glycolysis. Together with increased oxidative capacity, hypertrophy induced by physiological stimulus enhances both fatty acid and glucose oxidative metabolism.

Last but not least both thyroid hormones T3 and T4 are important nuclear transcription factors in cardiac plasticity, which contributes to remodeling of the heart and modulation of cardiac contractility and electrophysiological functions (Izumo et al., 1986; Nishiyama et al., 1998). The T3 upregulates expression of faster isoform of myosin heavy chain MyHCα, and downregulates expression of MyHCβ, which is generally accepted as more economical isoform of cardiac MyHC. Simultaneously, T3 upregulates expression of sarco/endoplasmic reticulum Ca^{2+} ATPase 2 (SERCA2). These changes and increased protein synthesis are considered as a beneficial and typical for the physiological concentric hypertrophy of the heart (Dillmann, 2010; Kenessey & Ojamaa, 2006; Pantos et al., 2008).

2.3. Pathological hypertrophy

Pathological cardiac hypertrophy represents enlargement of the ventricle causing reduced systolic and diastolic function due to dilatation of ventricle and thinning of the

ventricular wall in case of eccentric hypertrophy and drastically decreased volume of ventricle, fibrosis and dysregulation of ECM collagen network in case of concentric hypertrophy. This pathology often leads to HF and therefore is considered important risk factor for HF.

Conditions like myocardial infarction or ventricular dilatation cause eccentric pathological hypertrophy with preferred lengthening of cardiomyocytes over widening. If pathological conditions, as hypertension or valvular insufficiency result in cardiac remodeling, cardiomyocytes grow in thickness more than in length, increasing free wall and septum thickness, causing concentric hypertrophy and decrease in ventricle diameter (Grossman et al., 1975).

During adaptive or compensatory phase of pathological hypertrophy, cardiac function is maintained and hypertrophy is considered as physiological. Sustained pressure promotes transition from adaptive to maladaptive phase with several distinct characteristics. Mechanisms activating pathological hypertrophy are mechanotransduction and excessive neurohumoral signaling. The increased activities of angiotensin II and endothelin I or catecholamines belong to the main pathogenic signaling pathways (Paradis et al., 2000; Strand et al., 2006) despite their role in maintenance of contractility in the early adaptive phase of hypertrophy. However, if chronic stimulation becomes pathological neurohumoral signaling activates Gq proteins and phospholipase C (PLC) which leads to elevation of intracellular Ca^{2+} and activation of calcineurin and nuclear factor of activated T-cells (NFAT) signaling and calmodulin-dependent protein kinase II (CaMKII) signaling. NFAT is translocated upon activation into nucleus and activates expression of pro-hypertrophic genes (Wilkins & Molkentin, 2004). Intracellular release of Ca^{2+} is also activated by mechanosensitive transient receptor potential channels (TRPC1 and TRPC6) (Kuwahara et al., 2006; Seth et al., 2009).

Cardiomyocyte –ECM connection is mediated by heterodimeric membrane proteins integrins, bound intracellularly to a complex of kinases (focal adhesion kinase (FAK), integrin-linked kinase (ILK)) (Laser et al., 2000). Integrins mediated mechanotransduction activates phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K), protein kinase C (PKC),

Rho GTPase and ERK1/2 signaling (Laser et al., 2000). In contrast with physiological hypertrophy, pathological hypertrophy leads to a decrease in PCG-1α factor levels together with oxidative metabolism of fatty acids enzymes, therefore creating imbalance between progressing growth of the tissue and stopped mitochondrial biogenesis (Garnier et al., 2003; Sack et al., 1996).

In comparison with trained rats of similar degree of hypertrophy discussed above, 15 and 25 weeks old SHR did not show any improvement of oxidative capacity. Furthermore, mitochondrial fatty acid oxidation decreased by 14% and similarly PPARα, mCPTI and MCAD expression decreased or did not show a change in heart of 15 week old SHR (Rimbaud et al., 2009). To investigate glucose utilization of hypertrophied hearts of SHR relatively to healthy animals, Iemitsu et al. studied expression of key glycolytic enzymes phosphofructokinase and lactate dehydrogenase and Rimbaud et al. studied expression of GLUT4 and PDK4. Phosphofructokinase and lactate dehydrogenase were upregulated in hypertrophy induced by hypertension (Iemitsu et al., 2003). PDK4, kinase negatively regulating pyruvate dehydrogenase, and GLUT4 showed significant downregulation in SHR (Rimbaud et al., 2009). These different metabolic adaptations suggest stimulus specific response of cardiomyocyte metabolism. Shift from lipid to glucose oxidation in pressure overload model of hypertrophy (reviewed by Finck & Kelly, 2002) mimics neonatal cardiac metabolism, but significant substrate preference of hypertrophied heart was not confirmed.

Pathological hypertrophy is also characteristic by major ECM remodeling. Mechanical stress in volume overload, growth factors, insulin, cytokines and rennin-aldosterone signalization leads to an increased collagen production by fibroblasts. Decreased myocardial compliance and diastolic function is not caused by the change in total collagen content solely, but rather by shift in collagen types ratios and collagen network organization (Mukherjee & Sen, 1990). This complexity in remodeling is achieved by involvement of matrix metalloproteinases (MPP) degrading ECM components (Polyakova et al., 2004).

2.4. Creatine kinase

Creatine kinase (CK) system plays an important role in maintaining energetic homeostasis in myocardium as temporal and spatial energy buffer. CK was at first described as enzyme replenishing ATP near sites of energy utilization by catalyzing transfer of phosphate group from phosphocreatine (PCr) to adenosine diphosphate (ADP). PCr is a sole phosphagen in vertebrates and serves as an energy carrier between subcellular compartments of energy creation and utilization.

ATP replenishment capacity of CK significantly exceeds maximal ATP utilization and ATP regeneration by glycolysis and oxidative phosphorylation. CK in cardiac muscle shows maximal rate of ATP regeneration at 30 μmol/s per g, while maximal rate of ATP synthesis by oxidative phosphorylation reaches 2,5 μmol/s per g. Therefore, continuous and constant replenishment of ATP during cell's activity by CK is required to prevent significant fluctuations of intracellular ATP levels. Function of CK system results from CK equation and it's coupling to ATP hydrolysis (equation 1) (John A. Bittl et al., 1987).

$$MgADP^- + PCr + H^+ \leftrightarrow MgATP^{2-} + Cr$$

Equation 1: CK system reaction equation. PCr – phosphocreatine, Cr – creatine.

Concluding from the CK system reaction, regeneration of ATP in close proximity of ATPases is not the sole metabolic consequence of CK reaction. CK system also utilizes protons from ATP hydrolysis and thus protects sarcomere from increased local acidity (Teixeira & Borges, 2012). When observed from the opposite direction, CK reaction demonstrates ability to decrease rising ADP levels in site of energy consumption, especially during periods of intense workload. Increased ADP levels in vicinity of ATPases lead to the inactivation of ATPase. CK maintains high ATP/ADP ratio due to its low K_m for ADP (10-35 μM). Optimal ATP/ADP ratio then increases the thermodynamic efficiency of ATP hydrolysis. The maintenance of low ADP levels by CK reaction controls the glycolytic flux to some extent.

Another aspect of ADP accumulation is its trans-phosphorylation by adenylate kinase. This reaction yields ATP and AMP, which is converted into inositol monophosphate (IMP) and ammonia by AMP deaminase. Consecutive dephosphorylation of AMP and IMP by 5'-nucleotidase, yielding adenosine and inosine respectively, results in loss of the nucleotide pool. AMP and IMP are transported by the nucleoside transporters to the extracellular space and serve as important metabolic signaling molecules. In conclusion, suppression of ADP build-up in vicinity of ATP consuming enzymes stimulates the ATPases activity and prevents a loss of the adenine nucleotides (Aksentijević et al., 2010).

Aside from relatively low concentrations of ADP and ATP, importance of CK system as a spatial buffer is evident from mean diffusion lengths of ATP and ADP in comparison with PCr and Cr. This holds true especially in highly differentiated and excitable cells, with highly organized micro compartments. ADP with the mean diffusion length of only 1.8 μm is not sufficient energy carrier molecule for cardiomyocyte and would be limiting metabolite without PCr and Cr with mean diffusion lengths of 57 and 37 μm. High diffusivity of PCr is achieved by its smaller molecule, metabolic inertness in comparison with ATP and smaller charge in physiological pH (Wallimann et al., 1992).

2.4.1. Creatine kinase isoenzymes

CK system is composed of two cytosolic isoforms and one mitochondrial isoform of CK expressed in the myocardium. Cytosolic isoforms CKM (muscle) and CKB (brain) form functional dimers CKBB, CKMM and heterodimer CKMB (Eppenberg et al., 1967). Reaction site of mitochondrial CKs (sarcomeric) octamer is in intermembrane space of mitochondria, where CKs catalyzes phosphate transfer from ATP to PCr. Mitochondrial CKs can interact with inner and also outer mitochondrial membrane. Coupling of both membranes by CKs is considered the most favorable localization for CKs. CKs functionally coupled with ATP/ADP translocator (adenine nucleotide translocase, ANT) sharing cardiolipin patches in inner membrane, trans-phosphorylating ATP to yield PCr and saturating ANT with ADP to allow antiport reaction. This local hydrolysis of ATP produced by oxidative phosphorylation regenerates ADP that is necessary for reaction of

complex V in respiration chain. CKs thus stimulate respiration and maintain mitochondrial membrane potential. Maintained respiration and membrane potential protects mitochondria from excessive reactive oxygen species (ROS) production (Meyer et al., 2006). PCr produced by CKs reaction is transported to the cytosol by voltage dependent anion channel (VDAC), associated with CKs at the outer membrane (Schlattner et al., 2009).

Cytosolic CK dimers are localized near sites of ATP consumption and utilize PCr supplied by diffusion from mitochondrial CKs to increase local ATP/ADP ratio while producing Cr that provides feedback about energy demand to mitochondria and indirectly stimulates oxidative phosphorylation (Wallimann et al., 1992). Major dimer in healthy adult myocardium is CKMM, with main portion soluble in the cytosol. Dominant portion of bound CKMM is found in M line of cardiomyocytes, functionally coupled with myosin ATPases, maintaining their high activity during increased work load through preferential metabolic flow substrate channeling (Gregor et al., 2003). Second functional aspect of CKMM localization in M line results from substrate availability. The movement of contracting thick filament lattice and interlinking thin filaments push the myofibrillar reaction products towards the M line, increasing availability of ADP and H^+ for CK.

Another binding site for CKMM is near sarcoplasmic reticulum, where CKMM is coupled with SERCA (Korge et al., 1993). Binding of CKM dimer is a part of the sarcomeric M line structure, but has dynamic character dependent on pH conditions (Zurmanova et al., 2007). Specific interaction of two pairs of lysine residues (K104/K115 and K8/K24) with binding partners of the M line was demonstrated for the CK molecule in central fragment of myomesin 1 (My7-8) and M-protein (myomesin 2, M-Pr6-8) (Hornemann et al., 2000).

CKBB represents only a minor portion in differentiated cardiomyocytes and it reaches its peak activity during neonatal development (Hall & DeLuca, 1975). CKBB interacts with cardiac α-actin of developing myotubes and it seems that CKBB is involved in myotube formation during myogenesis. It possess higher affinity to PCr which allows its activity under lower energetic state (Wallimann et al., 1992).

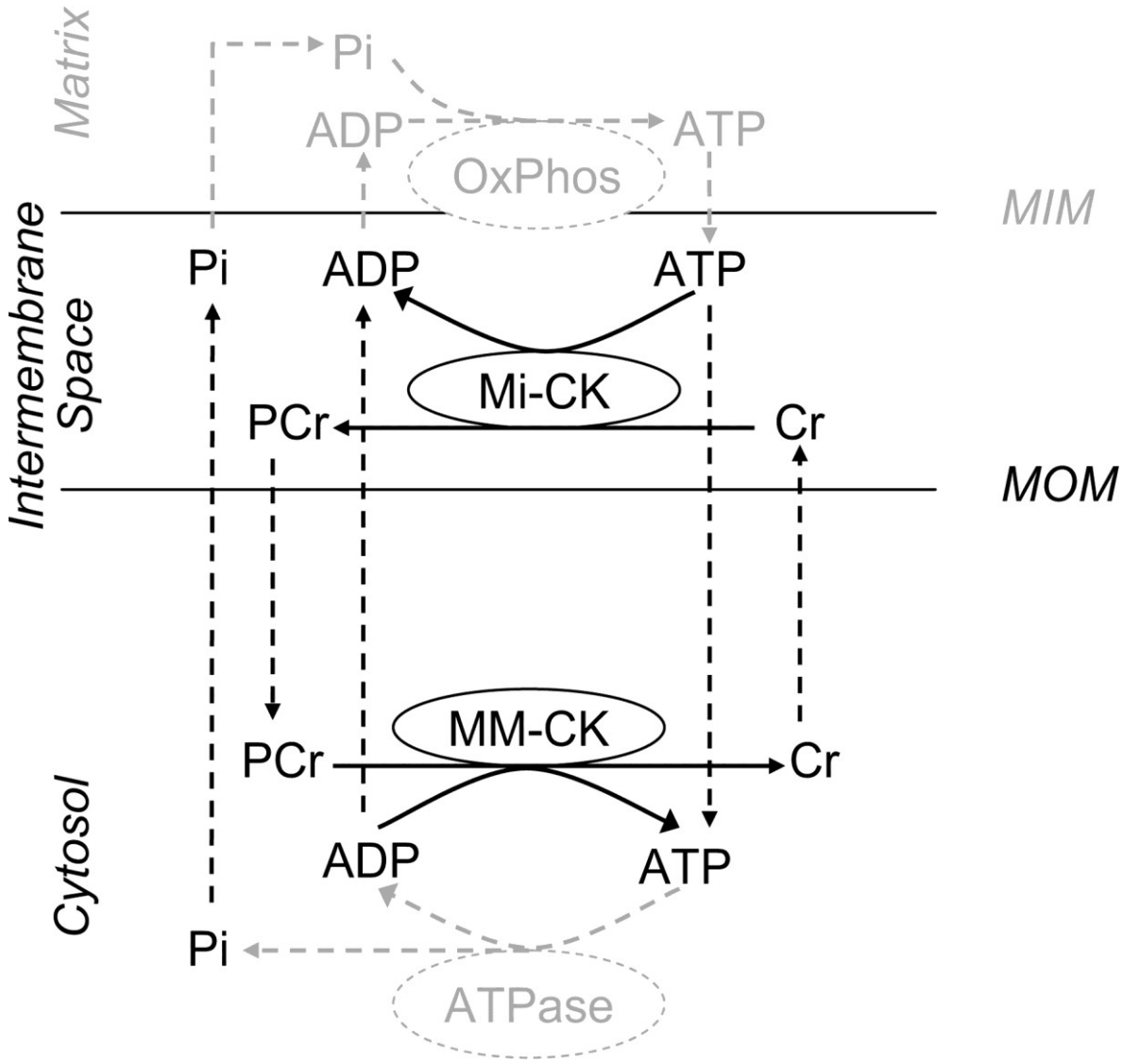

Fig. 2: *Scheme of the CK system compartmentalization in the cardiomyocytes. ATP produced by oxidative phosphorylation is transported through the mitochondrial inner membrane (MIM) to the intermembrane space, where CKs catalyzes reaction of ATP and creatine, yielding PCr and ADP. Restored ADP is transported back to the mitochondrial matrix and stimulates ATP synthase. PCr in the cytosol with higher diffusivity than ATP carries energy stored in phosphate group bonds to the sites of utilization – CK coupled with ATPases (adopted from (Zuurbier & van Beek, 1997)).*

Highly organized spatial localization of CK isoforms in cardiomyocytes is important for maintenance of energetic flows and proper function of the CK system.

Changes in intracellular organization and overexpression of structural proteins of the hypertrophied cardiomyocyte might play a leading role in the loss of function of the CK system, which may lose its binding site or spatial organization. These changes might contribute significantly to decrease in the pumping function of the heart, even with the minimal change in the level of the CK isoforms expression.

PCr/ATP ratio of approximately 1.8 represents energetic state of the healthy heart. Impairment of CK system contributes to the energy-starved state of myocardium in HF. Reduced PCr/ATP ratio has been shown in patients with cardiac dysfunction due to hypertension, obesity, type 2 diabetes and dilated cardiomyopathy, proposing a connection between cardiac function and energetic status (Kitzenberg et al., 2016).

As mentioned above, hypertrophic growth leads to hypoxic conditions, with severity dependent on level of the angiogenesis disruption. Increased expression observed in hypertrophy might play a protective role against hypoxia, because CKBB is more than capable to prevent anoxic tissue from complete depletion of ATP levels. This effect was demonstrated on transgenic mice liver expressing high levels of CKBB exposed to 40 minutes of ischemia and 90 minutes of hypoxia. Even with impaired mitochondrial function, CKBB maintained ATP levels and pH until considerable PCr depletion, indicating striking resistance to oxygen and/or nutrients deprivation in comparison with normal liver (Miller et al., 1993).

2.5. Myomesin

The sarcomere, as a fundamental functional unit of cardiomyocyte, requires complex cytoskeletal scaffold holding myosin and actin filaments in place. Each sarcomere is surrounded by two Z-lines, important for stability of sarcomere. Thick filaments of the sarcomere cross connect in the middle of the sarcomere, in the M-line of a region called H-zone. Proteins of the myomesin family belong between one of the main components of the M line.

Myomesin-1 (myomesin) and myomesin-2 (M-protein) are prominent members of myomesin family found in the central part of the sarcomere in cardiomyocytes. They are

encoded in humans by *Myom1* and *Myom2* genes respectively. Myomesin, 185 kDa protein (Grove et al., 1984), is expressed in each type of striated muscle and consist of 12 conserved immunoglobulin-like (Ig) and fibronectin type III (Fn) domains. N-terminal domain of myomesin (My1) and its binding of myosin are well described (Bantle et al., 1996; Vinkemeier et al., 1993).

More recent studies revealed that myomesin creates anti-parallel dimers, cross-linking and stabilizing myosin filaments in the M-band similarly to a role of an α-actin in Z-line of the cardiomyocyte (Lange et al., 2005). Central part of the myomesin is involved in interaction with m4 titin domain (Obermann et al., 1997) and My7-8 domains of myomesin bind with CKMM lysine residues (K8, K24, K104 and K115) (Hornemann et al., 2003). Interaction of myomesin and CKMM is pH dependent with dynamic association and dissociation, which might represent energy state of the cardiomyocyte. Dissociation constant of myomesin/CKMM at pH 6.8 is in the range of 50-100 nM (Hornemann et al., 2003). Interestingly, substrate channeling between CK and myosin ATPase reached maximal rate also under slightly acidic pH (Gregor et al., 2003).

As a result of an alternative splicing, additional 96 amino acids long elastic EH segment can be inserted between My6 and My7 domains (Schoenauer et al., 2011). EH-myomesin is the main myomesin isoform in the embryonic heart (Agarkova et al., 2000) and it was also shown to be upregulated in failing heart model of dilated cardiomyopathy (Schoenauer et al., 2011). In this study by Schoenauer et al., 41-fold upregulation of EH-myomesin expression in dilated cardiomyopathy patients was correlated with a decrease in cardiac function. Myomesin appears to be an essential structural component of the sarcomere, since its localization is consistent from early assembly of first myofibrils to maturity.

2.6. Hexokinase

Hearts natural response to the increased energetic overload and rising ADP/ATP ratio is stimulation of the glycolysis. The response to impaired energetic homeostasis in decompensation phase of the hypertrophy is even greater stimulation of glycolysis on expression level (Gottlob et al., 2001). Hexokinase (HK) is one of the key enzymes in

glycolysis and glucose uptake control. HKs catalyze endogenic phosphorylation of glucose to glucose-6-phosphate (G-6-P), the first and irreversible step in the glucose metabolism. Phosphorylation of the glucose to G-6-P maintains concentration gradient required to facilitate the glucose uptake by glucose transporter 1 (GLUT1) and glucose transporter 4 (GLUT4). G-6-P is afterwards utilized in consecutive steps of the glycolysis, pentose phosphate pathway, glycogenesis or hexosamine biosynthetic pathway (Wilson, 2003).

Mammals express four isoforms of HK, namely HK1-4. Highly specific isoform HK4 – glucokinase – is expressed primarily in liver and pancreas. The other HK isoforms have molecular weight approximately 100 kDa. HK2 possesses two catalytic domains at both N and C terminal domains however HK1 and 3 have only one catalytic site at C terminal domain of the molecules. Myocardial HKs are expressed in two major isoforms, ubiquitous HK1, largely bound to mitochondria of healthy myocardium and HK2, tissue specific for skeletal and cardiac muscle and adipose tissue, localized (under physiological conditions) in the cytosol (Southworth et al., 2007). Minor HK3 isoform has ubiquitous expression and is not dominant in any tissue. The HKs are constitutively active kinases and the changes in the expression directly regulate glucose metabolism on the cellular level.

Increased expression of CK and HK isoforms occurs in mild hypoxia and HK1 and 2 isoforms have anti-apoptotic effect by increased binding to outer mitochondrial membrane in severe hypoxia (Waskova-Arnostova et al., 2015). HKs bind to mitochondrial membrane via the VDAC (Anflous-Pharayra et al., 2007). Mitochondrial binding motif at N-terminal domain (21 amino acid sequence in hydrophobic α helix) was found in HK1 and 2, but not in HK3 (Sui & Wilson, 1997).

Decreased susceptibility of mitochondria to apoptosis was best described in context of HK2 binding to the outer membrane. HK2 association with VDAC is regulated by phosphorylation of Thr473 by Akt, which is preserved in rat, mouse and human. This phosphorylation site for Akt was found in HK2 but not HK1 and HK3 isoforms. Increase in mitochondrially bound HK2 after phosphorylation by Akt is supported by several studies, where activation of Akt leads to an inhibition of permeability transition pore (PTP) opening. Binding of HK to the VDAC prevents its closure, increases availability of ADP

for F_0F_1-ATPase through adenine nucleotide transporter (ANT) interacting with VDAC and therefore couples the glucose metabolism and ATP synthesis, which in turn helps with maintenance of membrane potential and reduction of ROS production (da-Silva et al., 2004; Gottlob et al., 2001).

Another proposed cardioprotective property of HK – mitochondria interaction via binding to the mitochondrial PTP component of VDAC is its inhibition of binding of the proapoptotic proteins of Bcl-2 protein family, Bcl-2 associated X protein (Bax) for instance, and prevention of PTP opening and consecutive release of cytochrome c (Anflous-Pharayra et al., 2007; Eskes et al., 1998; Shoshan-Barmatz et al., 2009; Waskova-Arnostova et al., 2015).

Promotion of HK - mitochondria association is not the only role of an activated Akt due to cardiac hypertrophy. Akt is a serine/threonine kinase with diverse functions in regulation of glucose metabolism, growth and survival, activated by a wide range of receptors, including insulin receptor. Several studies also suggest correlation between Akt activity and HK2 expression, supporting possible transcriptional regulation of HK by activated Akt. Moreover, hyperactivity of a mTOR, an important downstream effector of Akt, increased expression of HK2 (Bhaskar et al., 2009), providing additional evidence of HK regulation by Akt pathway. Activated Akt, important downstream effector in cardiac hypertrophy, increased mitochondrially bound HK activity by 50% without any effect on the total HK activity (Gottlob et al., 2001).

Hypertrophied rat hearts adapted to severe intermittent hypobaric hypoxia show increased mitochondrial HK activity together with increased total HK expression (Waskova-Arnostova et al., 2015). Expression under hypoxic conditions is enhanced by hypoxia-inducible factor 1 (HIF-1) through a consensus motif for HIF-1 in the HK2 promoter (Riddle et al., 2000). To activate a transcriptional program, α subunit of HIF-1 has to be stabilized by the hypoxic conditions. Additionally, enhancement of transcription by HIF-1 is connected to Akt/mTOR pathway through complex 1 of the mTOR, which upregulates transcription and translation of HIF-1α subunit (Düvel et al., 2010). As cardiac hypertrophy can induce mild hypoxic conditions and disrupts energetic homeostasis, HK

association with mitochondria can appear as the response to pressure overload and subsequent hypertrophic signaling. Aside from antiapoptotic protective effect, HK2 directly attenuates cardiac hypertrophy. Overexpression models as well as HK2 knockouts provide evidence of protection against H_2O_2. The latter shows increased susceptibility to IR injury and pressure overload, while cardiac specific HK2 overexpressing transgenic mice show attenuated hypertrophy and reduced ROS levels (McCommis et al., 2013). ROS represent an important and widely effective mediator of cardiac hypertrophy, activating pathways as Ca^{2+}/calmodulin dependent kinase, NFAT and MAP3K (Takimoto & Kass, 2007). HK2 supplies G-6-P to the pentose phosphate pathway, forming NADPH reducing equivalent, necessary for glutathione peroxidase and reduction of glutathione, a potent antioxidant neutralizing ROS. Prevention of the ROS accumulation together with upregulation of the glucose metabolism in order to compensate the decreasing cardiac fatty acid metabolism are two direct mechanisms attenuating cardiac hypertrophy mediated by HK2.

2.7. Spontaneous hypertensive rats

Hypertension is a long term medical condition also known as high blood pressure. Hypertension is a major risk factor for stroke, HF, end-stage renal disease and coronary artery disease and with rapidly increasing worldwide prevalence represents an important public health challenge (He & Whelton, 1997; Kearney et al., 2005). As a complex cardiovascular disease with plethora of causes and symptoms, hypertension is also an aim of broad spectrum of studies. Hypertension is divided into essential hypertension, the most common cardiovascular disease with no identifiable cause, which develops due to polygenetic and lifestyle factors (reviewed by Cowley, 2006). Less prevalent secondary hypertension develops as a consequence of a secondary disease.

The most popular animal model for studies of essential hypertension and its symptoms is the spontaneous hypertensive rats (SHR) strain, which was bred out of Wistar Kyoto (WKY) strain rats, by breeding individuals with essential hypertension. SHR rats develop hypertension spontaneously from 5^{th} to 10^{th} week after birth and reach a plateau of increased systolic blood pressure levels between 10^{th} and 15^{th} week (Shimamoto et al.,

1982). Along with hypertension, SHR rats develop significant myocardial hypertrophy of left ventricle (LV) between 9 and 12 weeks after birth as result of chronic pressure overload from increased systolic pressure (Shimamoto et al., 1982). Studies suggest, that hypertension is not the only cause of this hypertrophy and that oxidative stress resulting from various mutations targeting metabolism, Krebs Cycle and enzymes of oxidative phosphorylation are another stimuli triggering hypertrophic signaling and growth (Alvarez et al., 2008).

Compensational hypertrophy in SHR rats observable at 4 months of life leads to a nonfailing pathological phase, with decreased contractility and fibrosis between 8 and 10 months of life (Bing et al., 1995). Decrease in contractility is attributed to impaired ATP turnover and thus depressed mitochondrial respiration. Cardiac myosin ATPase activity in 12 months old SHR rats showed 50% depression during hard work in comparison to WKY animals of the same age. Same study showed elevated superoxide production and greater lipid peroxidation in LV, suggesting mitochondrial dysfunction after intense work (Hickey et al., 2009).

Between the ages of 18 and 24 months, late stage of pathological hypertrophy causes ultimate transition to HF. This transition is accompanied by increased passive stiffness of papillary muscle and increased collagen concentration, interstitial fibrosis and impaired contractile function of the LV (Conrad et al., 1995). These mechanisms together with mitochondrial dysfunction are the key components of transition from compensated LV hypertrophy to HF. Adaptations in CK and HK activity and expression from early hypertrophy to late stage HF are well described (Waskova-Arnostova et al., 2015; Ye et al., 2001), but dynamics in its intracellular localization remain unknown and will be the subject of this thesis, possibly answering questions about CK and HK influence on cardiac metabolism in progress from mild hypertrophy to pathological stage.

2.7.1. Alterations in SHR metabolism

Rimbaud et al. provided comprehensive overview of developing changes in physiology, hypertrophic genes expression and metabolism observed in SHR rats. Threefold increase in brain natriuretic factor (BNP) and fivefold increase in MyHCβ and

atrial natriuretic factor (ANF) expressions were observed in 25 weeks old SHR, accompanying the most developed stage of hypertrophy (Rimbaud et al., 2009).

Expression of the modulatory calcineurin-interacting protein-1 (MCIP1) was increased by 110% relatively to control animals, indicating activation of calcineurin hypertrophic pathway in SHR animals (Rimbaud et al., 2009). As described in chapters above, hypertrophy induced by pressure overload leads to decreased expression of genes involved in fatty acid oxidation. However, it is important to mention that SHR animals possess a gene defect in CD36 – membrane transporter of long chain fatty acids, representing preferred substrate in adult myocardium (Hajri et al., 2001; Neely et al., 1972). Moreover, it was shown that alterations in fatty acid oxidation enzymes expression occur in SHR as a genetic defect specific for this model and not due to hypertension. At the age of 4 weeks with normal blood pressure, many of mitochondrial enzymes involved in long chain fatty acid oxidation are downregulated, resulting in decreased production of nicotinamide adenine dinucleotide (NADH) and reduced flavin adenine dinucleotide ($FADH_2$). It results in accumulation of lipid intermediates, lipotoxicity and enhanced ROS production (Labarthe et al., 2004; Meng et al., 2009).

Hypertrophied and mainly failing heart demonstrates shift from fatty acid oxidation to glucose metabolism. This was also observed in SHR animals. Expression of glycolytic enzyme alpha-enolase is increased in SHR already in 4 weeks of age (Meng et al., 2009). SHR also show elevation of three pyruvate dehydrogenase (PDH) subunits. Depressed expression was observed in two tricarboxylic acid cycle enzymes – succinate dehydrogenase and fumarate hydratase. Because of this depression, accumulation of succinate and fumarate may occur, which may promote anaerobic glycolysis (Jüllig et al., 2008).

Shift in glucose and fatty acid metabolism in SHR can affect insulin signalization and contribute to hyperinsulemia and insulin resistance, described for this experimental model. Insulin levels in SHR are 2,4 times higher than in WKY animals (Hajri et al., 2001). In comparison to control animals, SHR show also hypertriglyceridemia and glucose intolerance (Reaven & Chang, 1991). Metabolic changes vary between different tissues of

SHR. Decrease in myocardial metabolism is not as prominent, as is in liver tissue (Postnov et al., 2007).

Changes in the energy transfer in heart of SHR animals were observed. mtCK decreased by 22% in mitochondria of 20 months old SHR and activity of mtCK dropped by 50% in mitochondria of 18-months old SHR (J A Bittl & Ingwall, 1987; Jüllig et al., 2008). These findings are supported by P-31 nuclear magnetic resonance (NMR) study, suggesting 3 fold decrease in the flux through the CK system (Ingwall, 1984). CK efficacy was not altered in 15 weeks old SHR, but was significantly higher in 25 weeks old SHR in comparison to WKY (Rimbaud et al., 2009), suggesting shift in isoforms expression or CK localization. Similar redistribution of the relative amounts of CK isoforms in favor of the CKB monomer at the expense of the CKM monomer was described in renovascular hypertensive rats. Total amount of CK protein was not increased in comparison with the control animals, but both amounts of MB and BB dimmers increased significantly (Pauletto et al., 1989). Redistribution in favor of MB dimer was observed in SHR animals, with perfect correlation between increase in fetal CK isozymes and increase in ratio of ventricular weight to body weight (Ingwall, 1984).

In conclusion, results of expression and CK protein content distribution suggest characteristic CK isoforms distribution in compensated hypertrophy and in failing myocardium. Overall decrease in metabolism results in slower growth and lower bodyweight in comparison with 10 days old WKY animals. On the other hand, heart weight of SHR is significantly higher from the first postnatal day (Charvátová et al., 2012).

2.8. Adaptation to hypoxia

Hypoxia is a state of deprivation of an adequate oxygen supply, affecting different, often vital organs. Hypoxia develops in the environment of low partial oxygen pressure (pO_2), leading to an insufficient blood pO_2 (systemic hypoxia). Hypoxic conditions generally occur during ascend to a high altitude, or as a consequence of preterm birth due to an underdevelopment of the lungs.

Prolonged period of time spent in hypoxic environment results in an adaptation, characteristic by structural and metabolic adjustments of cardiovascular system. First reports of this effect in people were published in 1960 by Hurtado, who described lower incidence of myocardial infarction in people living at high altitude. Only two years before Hurtado, in 1958, Kopecky and Daum published fist experimental study describing cardioprotective effect of an adaptation to hypoxia (Hurtado, 1960; Kopecký & Daum, 1958).

Cardiovascular adaptation to chronic hypoxia can be also described as an effort to maintain homeostasis under condition of low oxygen supply, which naturally leads to a higher cardiac tolerance to the damage resulting from acute oxygen deprivation that occurs during ischemia. In contrast to hypoxia, ischemia is a reduction of blood supply caused by interruption of coronary blood flow and thus the damage resulting from ischemia is not only consequence of an acute oxygen deprivation but also of shortage of nutrients and insufficient removal of metabolic wastes.

Following reperfusion can cause additional, even more severe damage – called reperfusion injury – resulting from sudden restoration of oxygen levels in the tissue. Reintroduction of blood flow causes increased production of ROS and calcium overload that leads to cardiac arrhythmias. Numerous cardioprotective effects of chronic hypoxia against IR injury and cardioprotective pathways activated by this adaptation are known and were reviewed in depth by Kolar and Ostadal (Kolář, 2004). Cardioprotective effect of chronic hypoxia was extensively studied by many authors, but a number of adaptive mechanisms remain undiscovered.

Hypoxic adaptation results in increased tolerance against acute ischemia, ventricular arrhythmias, contractile dysfunction and reduced size of damaged myocardial tissue after myocardial infarction. Various experimental models and protocols confirmed this protective effect and uncovered underlying details as significant sex differences in susceptibility of cardiac muscle to ischemia (Ostádal et al., 1984). Cardiomyocytes under hypoxic conditions adapt by strengthening systems responsible for energy homeostasis with higher sensitivity for O_2. One of the adaptations is elevating total CK activity and CK

protein expression after IR to support contractile function. Changes in compartmentalization of CK isoenzymes might serve as a protective adjustment to buffer energy supply for working sarcomeres during ischemia and IR. We expect this effect to be more significant in animals adapted to hypoxia, serving as one of the cardioprotective mechanisms of chronic hypoxia.

As already mentioned above, ROS produced by the respiratory chain during IR are the major factor causing cellular damage to myocardium. Increased expression of CK isoenzymes under hypoxic conditions and strengthened compartmentalization of CK near sites of energy production and consumption support cardiac metabolism. Reinforcement of cardiac metabolism through stimulation of oxidative phosphorylation by CK allows cardiomyocytes to reestablish contractility and reduce ROS production. Therefore, changes not only in CK expression, but also localization can contribute to increased tolerance of animals adapted to chronic hypoxia to IR injury, as was already observed in the past.

To stimulate the hypoxic conditions in the laboratory environment, two kinds of hypoxic chambers are utilized:

- normobaric chamber, with reduced O_2 content in the air and sustained air pressure – normobaric hypoxia
- hypobaric chamber, utilized to stimulate high altitude environment by decreasing air pressure in the chamber – hypobaric hypoxia

Intermittent hypobaric hypoxia (IHH) was used as an adaptation for this thesis. IHH is also referred as high altitude hypoxia because the pO_2 of the chamber corresponds to a certain altitude. Barometric pressure for this study was gradually increased up to the point corresponding to an altitude of 7000 m.

3. Aims of the thesis

This thesis follows three specific aims regarding localization of enzymes of the energetic metabolism during development of the cardiac hypertrophy.

3.1. Aim I

To examine the shift in CK isoenzymes colocalization with the sarcomere during early and late stages of the cardiac hypertrophy in comparison with healthy animals.

3.2. Aim II

To examine the shift in HK isoenzymes colocalization with mitochondria during early and late stages of the cardiac hypertrophy in comparison with healthy animals.

3.3. Aim III

To examine the shift in CK isoenzymes colocalization with the sarcomere after ischemia and IR in animals adapted to IHH in comparison with animals kept in the environment with normal barometric pressure.

4. Materials and methods

4.1. Experimental animals

Male WKY, Wistar and SHR rats obtained from Academy of Sciences were used in this thesis. Experimental animals selected for the part focused on hypertension were divided into two age groups of 4 and 13 months for SHR and WKY strains. Younger age group (4 months) consisted of 6 WKY and 6 SHR rats. Older age group (13 months) contained 8 SHR rats. After reaching required age, rats were euthanized using cervical dislocation without use of an anesthesia and hearts were fixed for immunofluorescent staining.

Adult male Wistar rats (330 – 610 g body weight (BW)) selected for the part focused on IHH and IR injury were divided into six groups. Distribution into the experimental groups is described in Table 1.

All animals used for the thesis had free aces to water and a standard laboratory diet. The study was conducted in accordance with the Guide for the Care and Use of Laboratory Animals, published the National Institute of Health (NIH Publication No. 85-23, Revised 1996).

Adaptation	n	Stab. (min)	Isch. (min)	IR (min)
Normobaric stab.	3	15	-	-
Normobaric isch.	5	15	10	-
Normobaric IR	2	15	10	15
Hypoxic stab.	3	15	-	-
Hypoxic isch.	5	15	10	-
Hypoxic IR	2	15	10	15

Table 1: *Experimental protocol for ischemic and IR animals.*

4.2. Adaptation to hypoxia

Hypoxic adaptation utilized for this thesis was chronic IHH corresponding to the altitude of 7000 m. Wistar rats were exposed to IHH for 8 h/day, 5 days/week, with barometric pressure (p_b) lowered stepwise to the point equivalent to an altitude of 7000 m (p_b = 308 mmHg, 41 kPa, pO_2 = 65 mmHg, 8,6 kPa). This altitude was reached after 13 exposures and total number of exposures was in the range of 24 – 30 exposures. The control group of animals was kept at the pressure corresponding to an altitude of 200 m (p_b = 742 mmHg, 99 kPa, pO_2 = 155 mmHg, 20,7 kPa).

4.3. Ischemia and IR injury

Animals in ischemic and IR subgroups from both hypobaric and normobaric groups were subjected to myocardial IR one day after the last exposure. Animals were anesthetized with intraperitoneal injection of sodium pentobarbital (60 mg/kg of bodyweight). To ensure proper ventilation during surgical procedure, a tracheotomy and intubation with a cannula connected to a rodent ventilator was performed. Rats were ventilated with room air at 65 - 70 strokes/min (tidal volume of 1,2 ml/100 g body weight). Animals were placed on a heated table to maintain the rectal temperature between 36,5 and 37,5 °C.

Access to the thoracic cavity was gained by left thoracotomy. Pericardium was removed and a poly-ester ligature 6/0 was tied around the left anterior descending coronary artery. The ligature was placed 1 – 2 mm distal to the origin and an occlusive snare was placed around it. A polyethylene tube was pulled on the suture, close to the occlusion. After 15 minutes of stabilization, ischemia was induced by tightening of the suture around the coronary artery. Duration of the occlusion was 10 minutes and following reperfusion in IR group lasted 15 minutes. Dissected heart was then fixed using Langendorff assay as described below.

4.4. Sample preparation

4.4.1. Tissue fixation

Langendorff assay was prepared in dissecting room. Two perfusate reservoirs were filled with relaxing calcium free Tyrode's solution (in mM: 140 NaCl, 5,4 KCl, 1 Na_2HPO_4, 1 $MgCl_2.6H_2O$, 10 glucose, 5 HEPES, pH 7,4) and 4 % paraformaldehyde respectively, both freshly prepared day before. Reservoirs were placed high enough above heart placement, to ensure appropriate hydrostatic pressure for perfusion under constant pressure. Inflow reservoir tubes were closed using hemostats for switching between perfusates.

Six WKY and six SHR rats were euthanized by cervical dislocation without use of anesthesia. Skin was cut under the last rib immediately after euthanasia and chest cavity was opened by two lateral cuts lead cranially through ribs. Heart with aortic trunk was dissected with single cut. Heart held with forceps was slipped over cannula attached to the outflow of reservoirs through ascending aorta. Surgical clamp was used to hold heart on cannula initially and was replaced by surgical thread before perfusion by Tyrode's solution (two minutes of perfusion) followed by paraformaldehyde perfusion for another 3 minutes.

Heart was removed from Langendorff assay, atriums and arteries were removed and disposed using razor. Ventricles were separated on left and right with septum and placed in closed flask with 10 ml of 4 % paraformaldehyde. After two hours of fixation in paraformaldehyde, solution was replaced by 10 % sucrose in miliporQ water and left in 4°C overnight. Left and right ventricles were briefly dried on a filter paper and frozen in the liquid nitrogen and stored in the -80°C.

4.4.2. Sectioning frozen tissue

Leica CM3050 S cryostat (Leica-microsystems, Wetzlar, Germany) equipped with microtome was used for sectioning of LV heart tissue. Cryostat was set on the temperature of -22°C. Specimen and equipment (cryostat blades, forceps, specimen disc and glass) were placed inside cooled cryostat overnight before sectioning, to ensure consistent temperature of equipment and specimen.

LV was mounted on specimen disc inside cryostat using Sakura Tissue Tek mounting medium. After sufficient freezing of the mounting medium, specimen disc was clamped tightly to a microtome arm. Disc was oriented in a way that the apex of the ventricle pointed straight down and the surface of pericardium was parallel with cutting blade.

Specimen was sectioned using the automatic preprogrammed sectioning to ensure regular speed and therefore cleaner cuts. Thickness of sections was 6 μm and only sections from myocardium (after cutting through pericardium) were used. Sections were collected from anti-roll plate on a warm glass slides (Menzel Glasser, Thermo Fisher Scientific). Two groups of specimen sections per each slide were encircled using wax marker, creating specimen wells. Slides were put in freshly prepared PBS buffer (pH 7.4) for rehydration.

4.4.3. Immunostaining

Slides were placed in a plastic staining cuvette filled with 100% methanol and incubated for 10 minutes in -20°C for permeabilization of the cell membranes. Slides were washed in PBS 3 times for 5 minutes. From this point further, slides were kept inside dark staining tray with high air humidity throughout the process of immunostaining.

To block non-specific binding sites of antibodies, 90 minutes of incubation in appropriate blocking buffer diluted in PBS containing 0,3 % Triton X-100 and 0,3 M glycine followed. Slides were washed in PBS 3 times for 5 minutes. Primary antibodies for CK M and CK B were diluted in 1:50 ratio with 1 % BSA in PBS buffer. Diluted primary antibodies were applied in volume of 100 μl per specimen well for 60 minutes. Slides were washed in PBS 3 times for 5 minutes.

Primary antibodies for myomesin 1 were diluted in 1:50 ratio with 1 % BSA in PBS buffer and applied in volume of 100 μl per specimen well for 60 minutes. Slides were washed in PBS 3 times for 5 minutes. Secondary antibodies for CKM and CKB (Alexa Fluor® 488) were diluted in 1:200 ratio with 1 % BSA in PBS buffer and applied in volume of 100 μl per specimen well for 60 minutes. Slides were washed in PBS 3 times for 5 minutes.

Secondary antibodies for myomesin 1 (Alexa Fluor® 647) were diluted in 1:200 ratio with 1 % BSA in PBS buffer and applied in volume of 100 µl per specimen well for 60 minutes. Slides were washed in PBS 3 times for 5 minutes. Excess PBS buffer was drained carefully and one drop of VECTASHIELD antifade mounting medium with 4', 6-diaminidino-2-phenylindole (DAPI) was applied on each specimen well. Slides were covered with cover slips and fixed by a thin layer of nail-polish applied along all edges of cover slip.

Similar protocol was used for immunostaining of HK-OXPHOS. Methanol with addition of 1 % SDS was used for the cell membrane permeabilization. Slices were incubated in appropriate blocking serum with unconjugated secondary antibodies for 90 minutes. Primary antibodies were diluted in 1 % BSA PBS in 1:100 ratio. Rabbit polyclonal primary antibodies were obtained from Abcam (antiHK1, antiHK2 and antiHK3). Mitochondrial marker primary antibody Oxphos BlueNative was obtained from Abcam. Anti-rabbit IgG Alexa Fluor® 488 conjugated was used against HK primary antibodies and anti-mouse IgG Alexa Fluor® 647 conjugated was used against mitochondrial marker.

4.4.3.1. Immunostaining for phalloidin colocalization

Because we used phalloidin as a marker for the M line to study effects of ischemia and IR injury on CK system localization in hypoxic and normal rats instead of myomesin, protocol for immunostaining slightly varies from the protocol above. Incubation with myomesin primary and secondary antibodies were replaced by 20 minutes of incubation with phalloidin (Alexa Fluor® 647 Phalloidin) diluted in 1:80 ratio with PBS buffer after the incubation with CK secondary antibodies.

4.4.4. Immunofluorescent microscopy

Images for this study were acquired using wide-field fluorescence microscope Olympus IX-81 (Olympus, Hamburg, Germany) and Cell-R live cell imagining system, 100x PlanApochromat objective (NA = 1,4), Hammamatsu Orca/ER digital camera, GFP

filter block U-MGFPHQ, excitation maxima 488 nm, emission maxima 507 nm and RFP filter block U-MWIY2 with excitation maxima 545-580 nm and emission maxima 610 nm.

Ten images of each sample well were taken using automated program in Experiment manager provided by Olympus Cell-R software. Regions of interest were chosen in random, properly sectioned and stained areas (requirements for selection were relaxed sarcomere and single layer of cells without precipitation of antibodies). Each position was optically sectioned at 0.5 μm steps resulting in ~12 focal planes.

4.4.5. Image analysis

4.4.5.1. Colocalization of CK and myomesin in SHR images

Quantitative analyses of the images were performed using Olympus Soft Imaging Solutions and FIJI software. First, images were deconvolved using the No Neighbor algorithm of Olympus Soft Imaging Solutions software. The background subtraction (rolling ball 5) was applied on green channel (CK) and focal planes were merged into one image, using FIJI software ZProjection function with selected Average Intensity projection setting. To acquire sharp recognition of the M line, Contrast Limited Adaptive Histogram Equalization (CLAHE, blocksize 127, histogram 256) was applied on the red channel (myomesin). Gaussian blur (sigma radius 12) was then applied on the duplicate of red channel, which was afterwards subtracted from the original image using FIJI's Image Calculator. Manders split coefficient used for colocalization analysis was calculated using ICA plugin.

4.4.5.2. Colocalization of HK and OXPHOS in SHR images

Quantitative analyses of the images were performed using Olympus Soft Imaging Solutions and FIJI software. First, images were deconvolved using the No Neighbor algorithm of Olympus Soft Imaging Solutions software. The background subtraction (rolling ball 5) was applied on both channels. Pearson's correlation coefficient was calculated using ICA plugin (cross hair = 3).

4.4.5.3. Colocalization of CK and phalloidin in IRI images

Quantitative analyses and deconvolution was performed as in previous chapter. To create a mask with a fluorescent signal corresponding to the M lines of cardiomyocytes, focal planes of the red channel (phalloidin counterstaining actin filaments) were first merged into one image, using FIJI software, ZProjection function with selected Average Intensity projection setting. Image was then inverted and a few sarcomeres were selected and saved as a region of interest. Gaussian blur (sigma radius 12) was applied on duplicate of the green channel (CK), which was afterwards subtracted from the original image using FIJI's Image Calculator to reduce background noise. Manders M1 coefficient used for colocalization analysis in selected regions of interest was calculated using ICA plugin.

4.4.5.4. Statistical analysis

GraphPad Prism 7 was used for statistical analyses of acquired colocalization coefficients. Non-parametric Mann-Whitney t-test was used to compare paired groups. ANOVA and Bonferroni post-test were used to compare more than two experimental groups. Only data with p value of $p < 0.05$ were considered as statistically significant. Error bars in graphs represent ± SEM.

5. Results

5.1. Aim I

Within this aim we studied effect of two different developmental stages of cardiac hypertrophy induced by pressure overload on CK isoenzymes localization in sarcomeres of the left ventricle myocardium from 4 months old and 13 months old SHR in comparison with 4 months old WKY rats.

5.1.1. CK localization in hypertrophied heart of 4 months old SHR

Colocalization of CKM with myomesin, evaluated by Pearson's correlation coefficient, was significantly higher in 4 months old SHR group (PCC = 0.4929) than in age matched WKY animals (PCC = 0.3134). PCC was increased by 57 % in SHR group. Colocalization of CKB with myomesin did not vary significantly between 4 months old SHR and WKY (PCC = 0.2122 for SHR and 0.2079 for WKY).

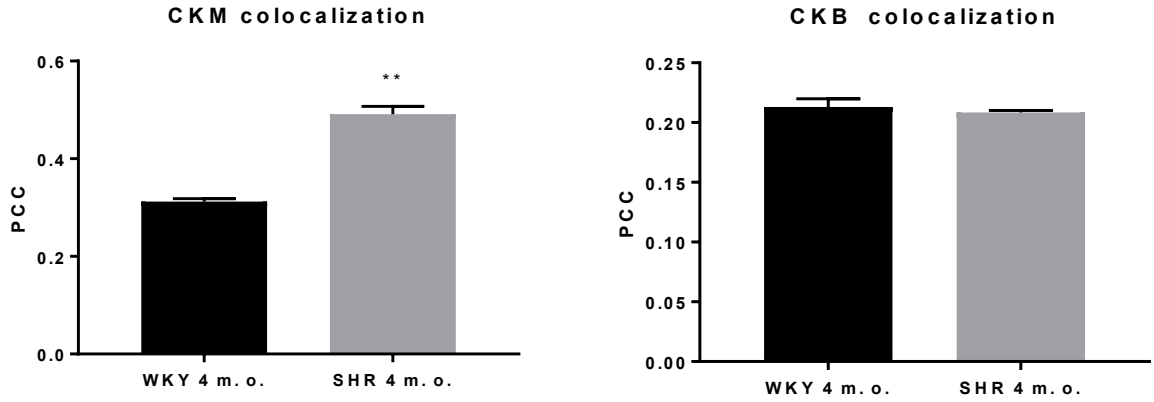

Fig. 3: *Colocalization of CKM (left) and CKB (right) with myomesin represented by Pearson's correlation coefficient (PCC). 4 months old WKY (n = 6) and 4 months old SHR (n = 6). Values are mean ±SEM (n = 4) P < 0.05.*

5.1.2. CK localization in hypertrophied heart of 13 months old SHR

Colocalization of CKM with myomesin, evaluated by Pearson's correlation coefficient, was significantly higher in 13 months old SHR group (PCC = 0.345) than in 4 months old WKY animals (PCC = 0.3134). Increase in favor of SHR group reached 10 %. Colocalization of CKB with myomesin was significantly higher in 13 months old SHR than in 4 months old WKY animals, reaching increase by 11 % (PCC = 0.2122 for WKY and 0.2363 for SHR).

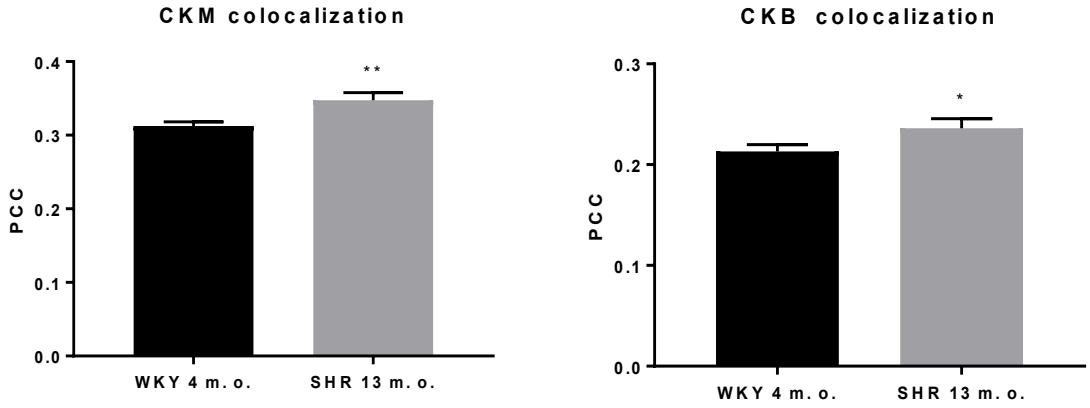

Fig. 4: *Colocalization of CKM (left) and CKB (right) with myomesin represented by PCC. 4 months old WKY (n = 6) and 13 months old SHR (n = 8). Values are mean ±SEM (n = 4) P < 0.05.*

Comparison across all three experimental groups revealed coordination between CK isoenzymes, localized in the M line. Increase in CKM colocalization with M line, significant in 4 months old SHR with developing hypertrophy, retreats in 13 months old SHR animals. Colocalization of CKM and the M line for this group revealed only 10 % increase in comparison with WKY. This decrease is accompanied by increased CKB, in the MB or BB dimer, taking place in the M line instead of muscle isoform.

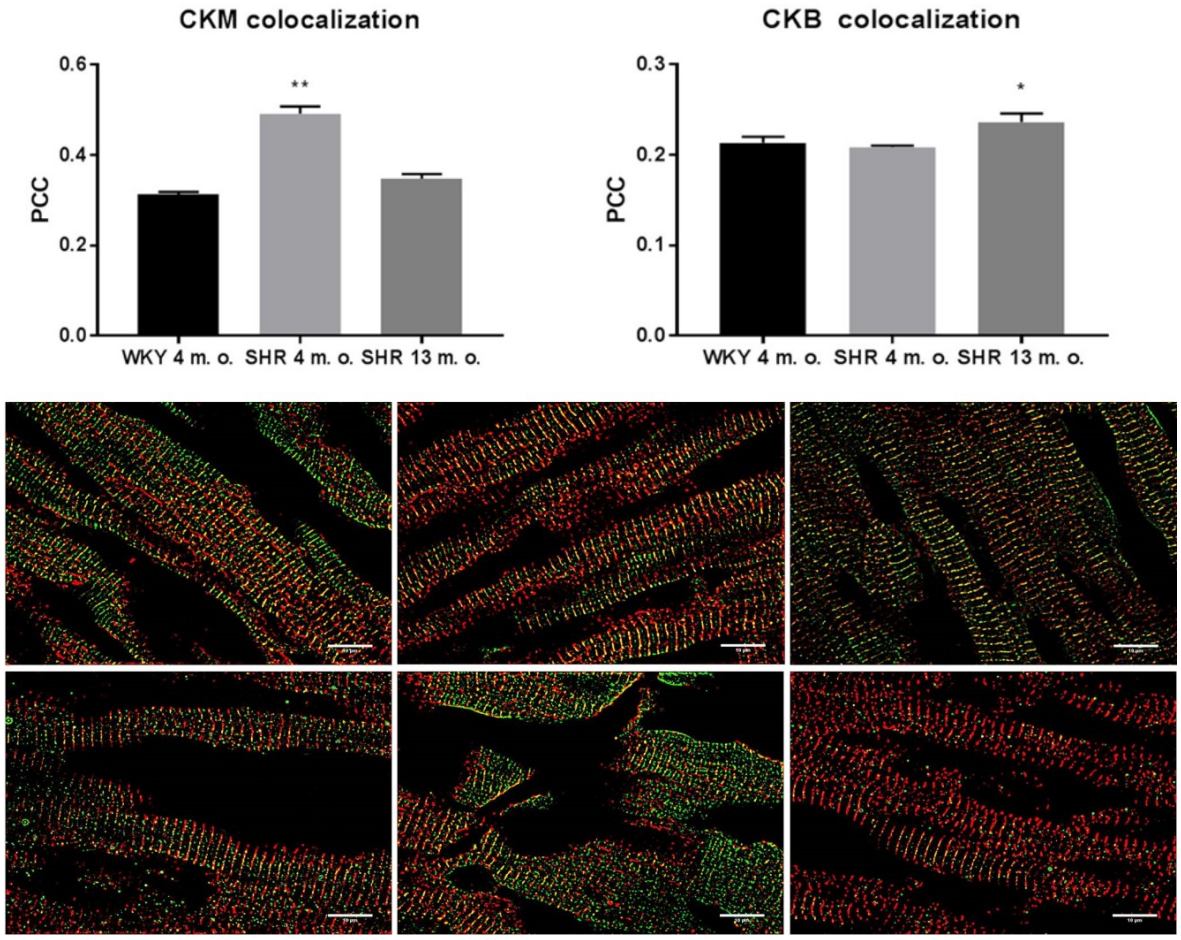

Fig. 5: *Colocalization of CKM (left) and CKB (right) with myomesin represented by PCC. 4 months old WKY (n=6), 4 months old SHR (n = 6) and 13 months old SHR (n = 8). Values are mean ±SEM (n = 4) P < 0.05 ** vs. other groups * vs. other groups. Representative images of longitudinal cryosections showing colocalization of CKM and myomesin (top row) and CKB and myomesin (bottom row) in LV of WKY 4 months old (left) SHR 4 months old (middle) and SHR 13 months old (right). Green color represents staining of CK and red color represents the myomesin staining.*

5.2. Aim II

Within this aim we studied colocalization of HK isoenzymes with mitochondria in the left ventricle myocardium from 4 months old and 13 months old SHR in comparison with 4 months old WKY rats.

5.2.1. HK1 - OXPHOS colocalization

Analysis of Pearson's correlation coefficient of HK and OXPHOS was used to determine association of HK with mitochondria. Colocalization of HK1 and OXPHOS enzymes was significantly higher in both SHR groups compared to WKY rats. Pearson's correlation coefficient of HK1 – OXPHOS was 0.69 for WKY (n = 6), 0.80 for SHR 4 months old (n = 6) and 0.80 for SHR 13 months old (n = 8), showing an increase by 16 % in both SHR groups.

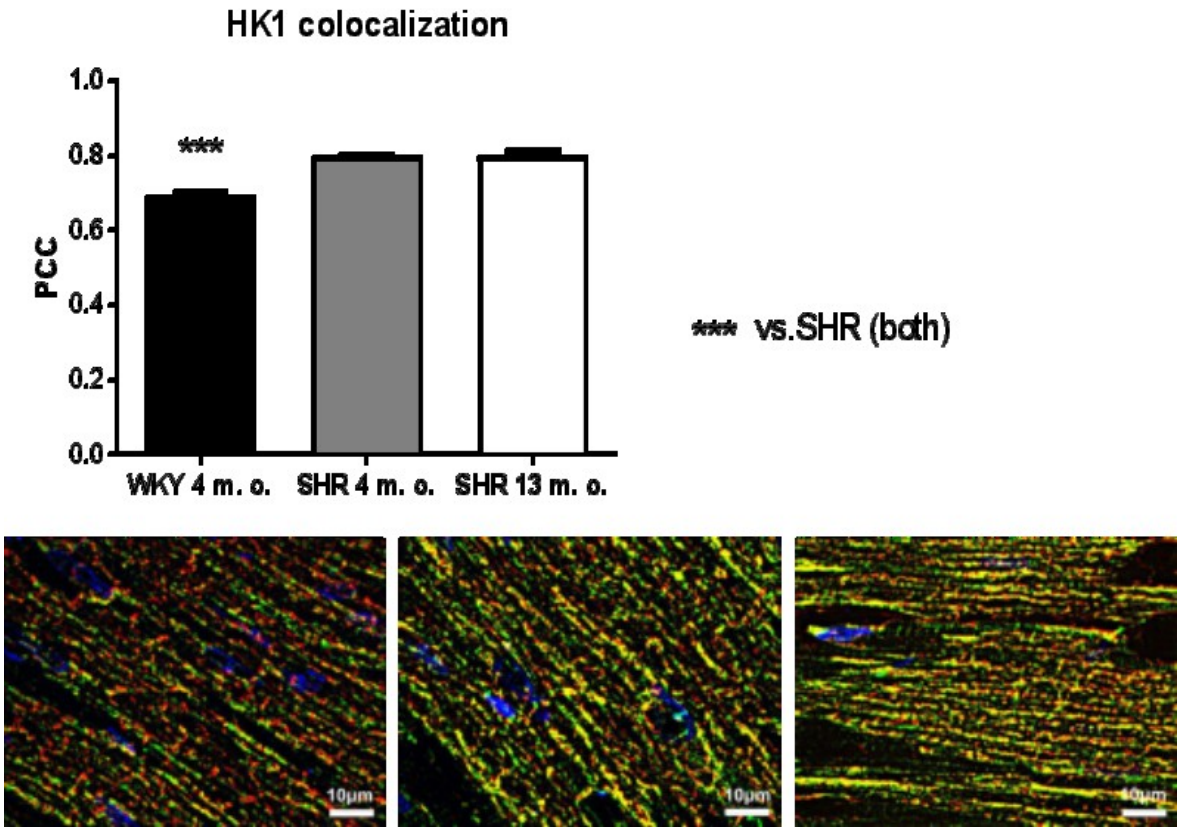

Fig. 6: *Colocalization of HK1 with OXPHOS represented by PCC. 4 months old WKY (n = 6), 4 months old SHR (n = 6) and 13 months old SHR (n = 8). Values are mean ±SEM (n = 4) P < 0.05. Representative images of longitudinal cryosections showing colocalization of HK1 and OXPHOS in LV of WKY 4 months old (left) SHR 4 months old (middle) and SHR 13 months old (right). Green color represents staining of HK and red color represents the OXPHOS staining.*

5.2.2. HK2 - OXPHOS colocalization

HK2 - OXPHOS colocalization did not change in 4 months old SHR relatively to the WKY controls (PCC = 0.67 for WKY and 0.71 for SHR 4 months old), but decreased significantly in 13 months old SHR (PCC = 0.49), by 27 % compared to 4 months old WKY animals. Degree of HK1 - OXHOPS and HK2 - OXPHOS colocalization did not differ for WKY animals. Colocalization of HK2 - OXPHOS was approximately one-fold lower than HK1 - OXPHOS in oldest SHR group.

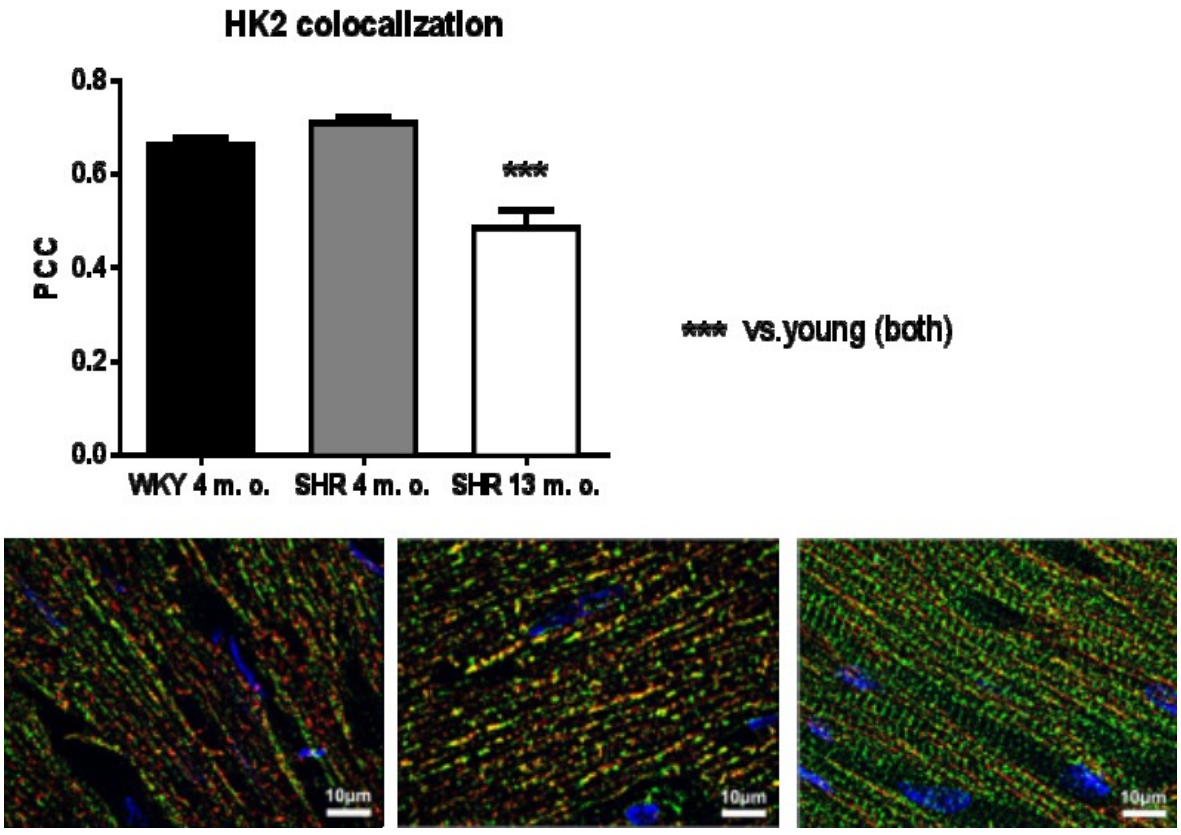

Fig. 7: *Colocalization of HK2 with OXPHOS represented by PCC. 4 months old WKY (n = 6), 4 months old SHR (n = 6) and 13 months old SHR (n = 8). Values are mean ±SEM (n = 4) P < 0.05. Representative images of longitudinal cryosections showing colocalization of HK2 and OXPHOS in LV of WKY 4 months old (left) SHR 4 months old (middle) and SHR 13 months old (right). Green color represents staining of HK and red color represents the OXPHOS staining*

5.2.3. HK3 - OXPHOS colocalization

HK3 - OXPHOS showed no difference between 4 months old WKY and SHR animals and highest colocalization between HK isoenzymes in both mentioned groups (PCC = 0.82 for both young groups). Colocalization was significantly lower in 13 months old SHR group than in both younger groups (PCC = 0.60). Amount of HK3 associated with mitochondria in 13 months old SHR in comparison with WKY controls decreased by 27 %.

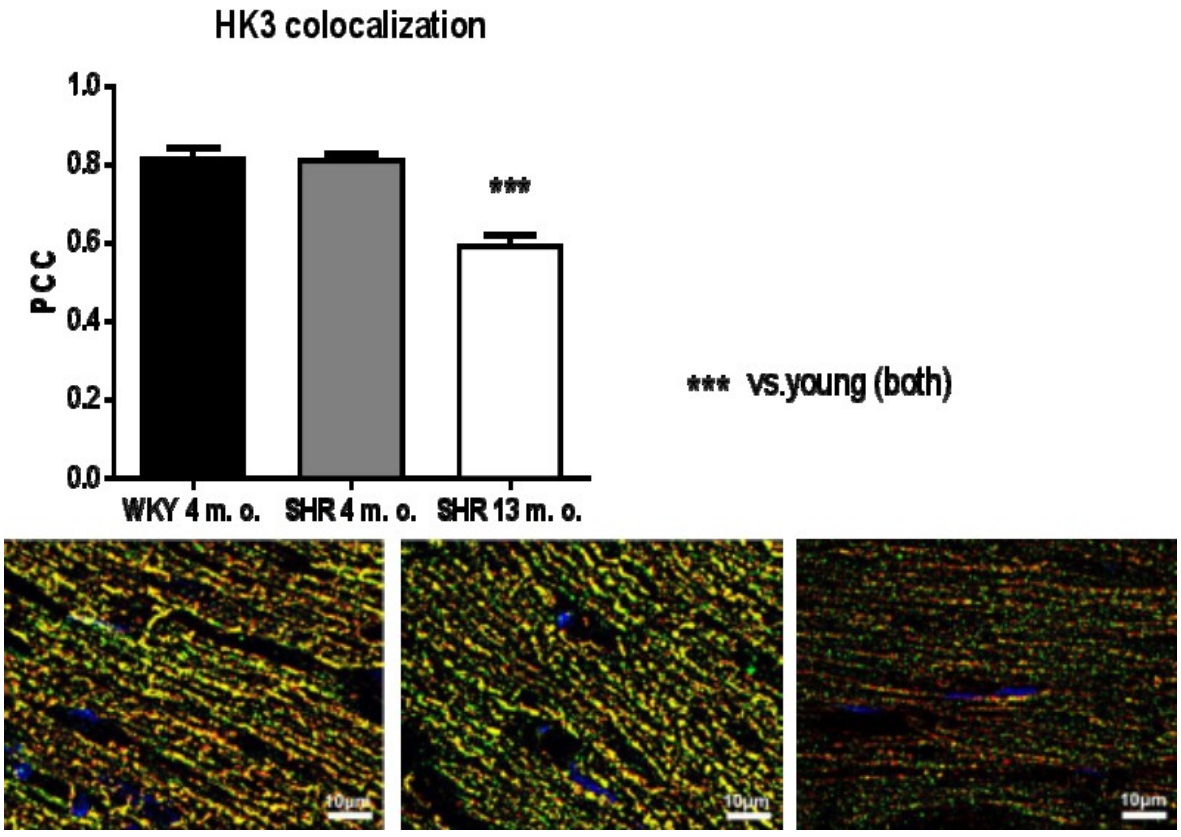

Fig. 8: *Colocalization of HK3 with OXPHOS represented by PCC. 4 months old WKY (n = 6), 4 months old SHR (n = 6) and 13 months old SHR (n = 8). Values are mean ±SEM (n = 4) P < 0.05. Representative images of longitudinal cryosections showing colocalization of HK3 and OXPHOS in LV of WKY 4 months old (left) SHR 4 months old (middle) and SHR 13 months old (right). Green color represents staining of HK and red color represents the OXPHOS staining*

5.3. Aim III

Within this aim we studied effect of short term IR injury on cytosolic CK isoforms location in cardiomyocytes of heart adapted to severe IHH.

5.3.1. Effect of hypoxia on CK localization

Difference in CKM - M line colocalization between IHH and control animals, expressed as Manders M1 coefficient, was not significant (M1 for hypoxic animals was 0.2566, n = 3 and for control animals 0.3438, n = 3). Values of M1 lower than 0.5 indicate weak colocalization of CKM and M line.

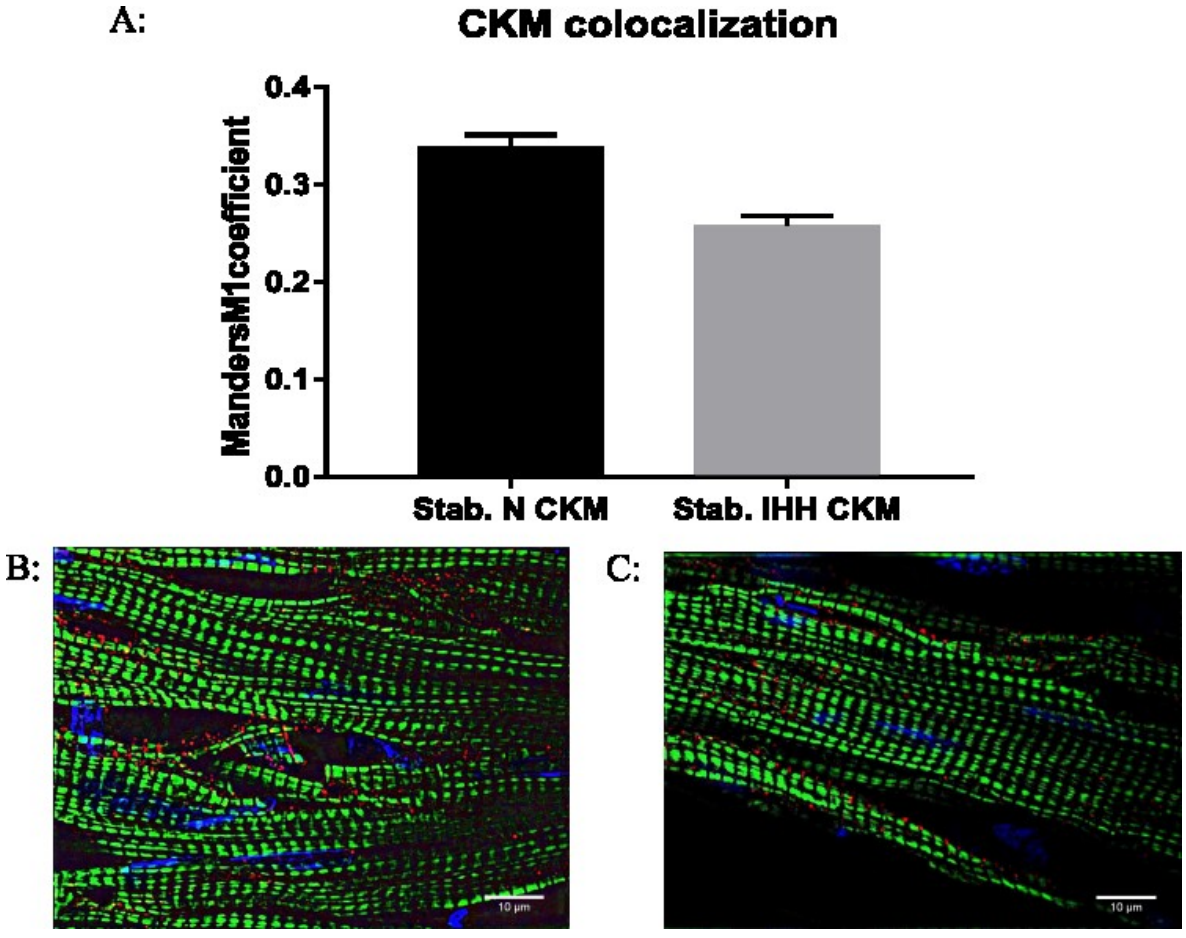

Fig. 9: *Colocalization of CKM with M lines represented by Manders' M1 coefficient (A). Values are mean ±SEM (n = 3). Representative images of longitudinal cryosections*

showing colocalization of CKM and actin with nucleus in LV of normobaric rat (B) and rat adapted to IHH (C). Green color represents staining of actin by phalloidin, blue color represents nuclear DAPI staining and red color represents the specific CKM staining.

Difference in CKM - M line colocalization between IHH and control animals, expressed as Manders M1 coefficient, was not significant (M1 for hypoxic animals was 0.178, n = 3 and for control animals 0.1325, n = 3). Value of M1 in this range of 0.1-0.2 shows weak colocalization of CKB and M line, therefore major portion of CKB is not in localized in the M line. This result is in accordance with the normal phenotype.

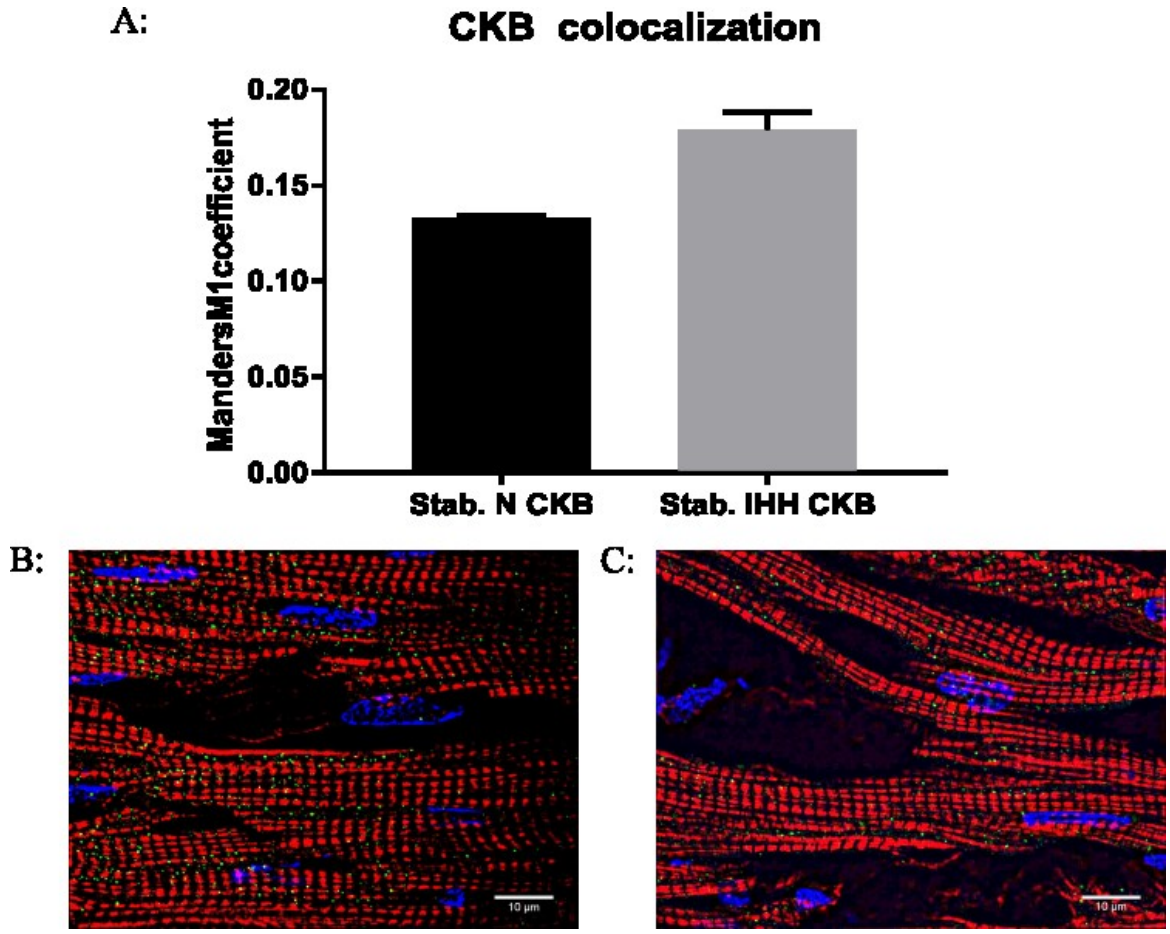

Fig. 10: *Colocalization of CKB with M lines represented by Manders' M1 coefficient (A). Values are mean ±SEM (n = 3). Representative images of longitudinal cryosections showing colocalization of CKB and actin with nucleus in LV of normobaric rat (B) and rat*

adapted to IHH (C). Red color represents staining of actin by phalloidin, blue color represents nuclear DAPI staining and green color represents the specific CKB staining.

5.3.2. Localization of CK after ischemia in hypoxic and normal hearts

IHH adapted animals showed significant decrease in localization of CKM in the M line after cardiac ischemia. Manders M1 coefficient revealed 80 % difference in colocalization of CKM – M line in IHH group. Normobaric control group showed balanced ratio of CKM signal overlapped with M line and signal outside M lines (M1 = 0.4825, n = 4), while IHH animals showed strong negative correlation of CKM with M lines (M1 = 0.08653, n = 4).

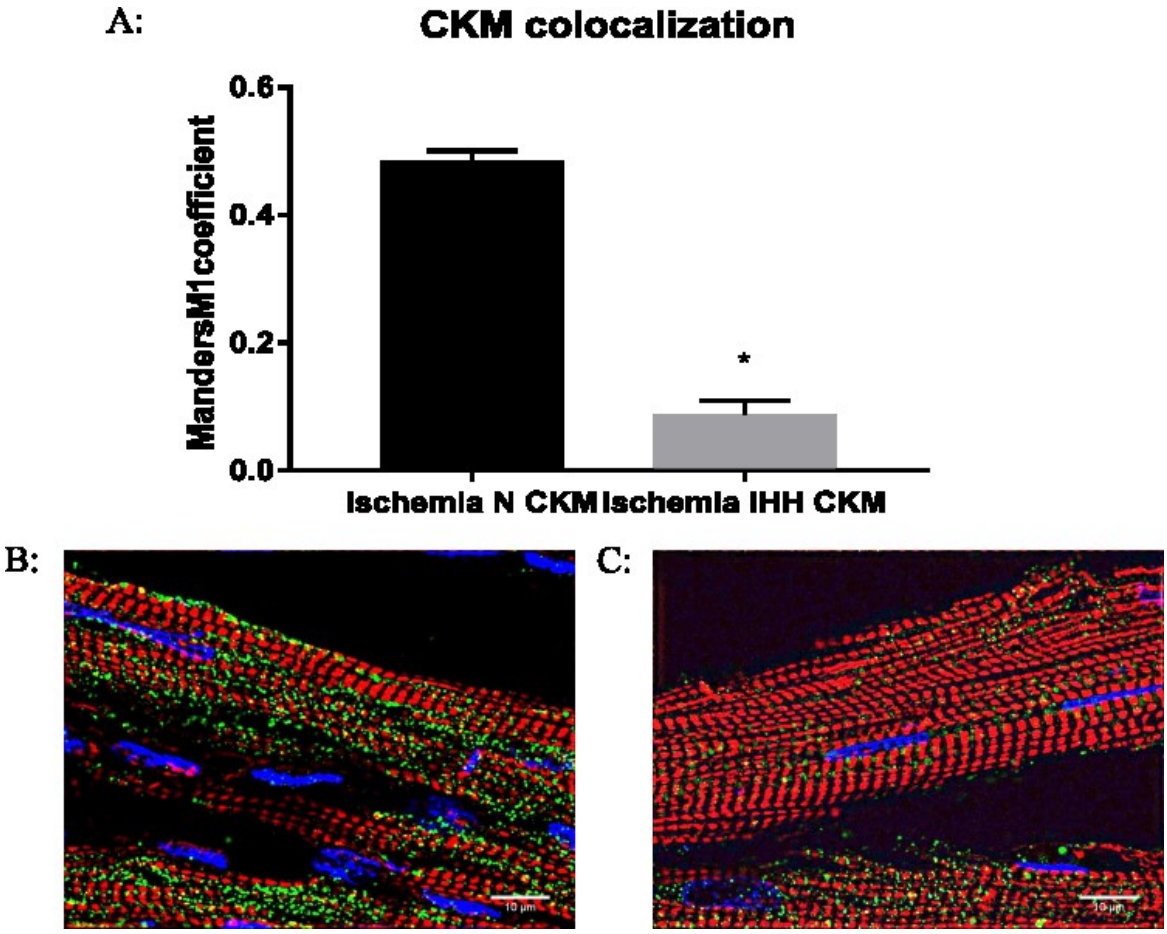

Fig. 11: *Colocalization of CKM with M lines represented by Manders' M1 coefficient (A). Values are mean ±SEM (n = 4) P < 0.05. Representative images of longitudinal cryosections showing colocalization of CKM and actin with nucleus of the LV myocardium*

after ischemia in normobaric rat (B) and rat adapted to IHH (C). Red color represents staining of actin by phalloidin, blue color represents nuclear DAPI staining and green color represents the specific CKM staining.

Similar difference was observed in CKB – M lines colocalization. Manders M1 coefficient of CKB – M lines in IHH group was significantly lower than M1 of control animals. Normobaric control group showed balanced ratio of CKB signal overlapped with and outside M lines (M1 = 0.5159, n = 4), while IHH animals showed strong negative correlation of CKB with M lines (M1 = 0.1486, n = 4).

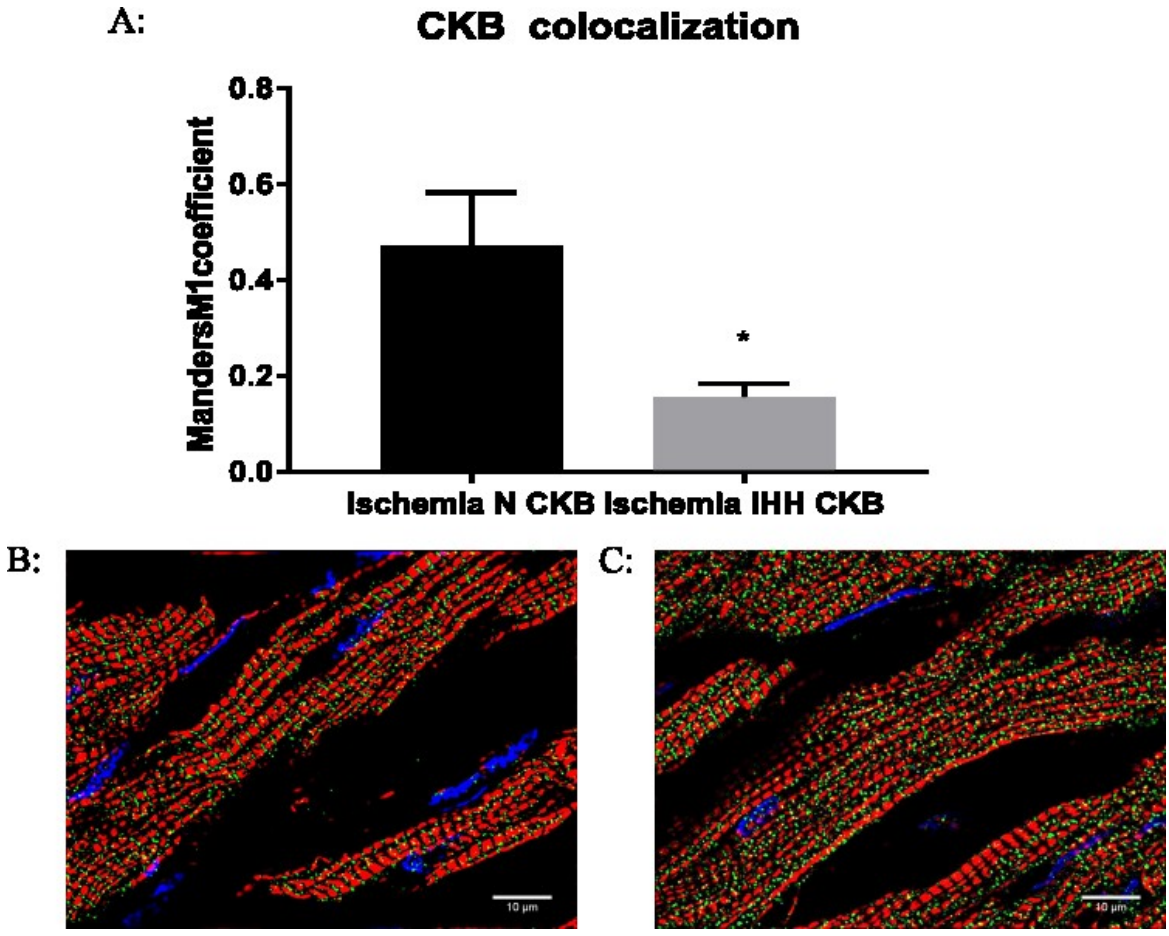

Fig. 12: *Colocalization of CKB with M lines represented by Manders' M1 coefficient (A). Values are mean ±SEM (n = 4) P < 0.05. Representative images of longitudinal cryosections showing colocalization of CKB and actin with nucleus in the LV myocardium after ischemia of normobaric rat (B) and rat adapted to IHH (C). Red color represents*

staining of actin by phalloidin, blue color represents nuclear DAPI staining and green color represents the specific CKB staining.

5.3.3. Localization of CK after IR in hypoxic and normal hearts

We did not observe any significant difference in CKM localization between animals adapted to IHH and normobaric control after IR injury. Values of Manders M1 coefficient suggest weak negative correlation of CKM (M1 = 0.3313, n = 3 and 0.337, n = 3 for control and IHH groups respectively).

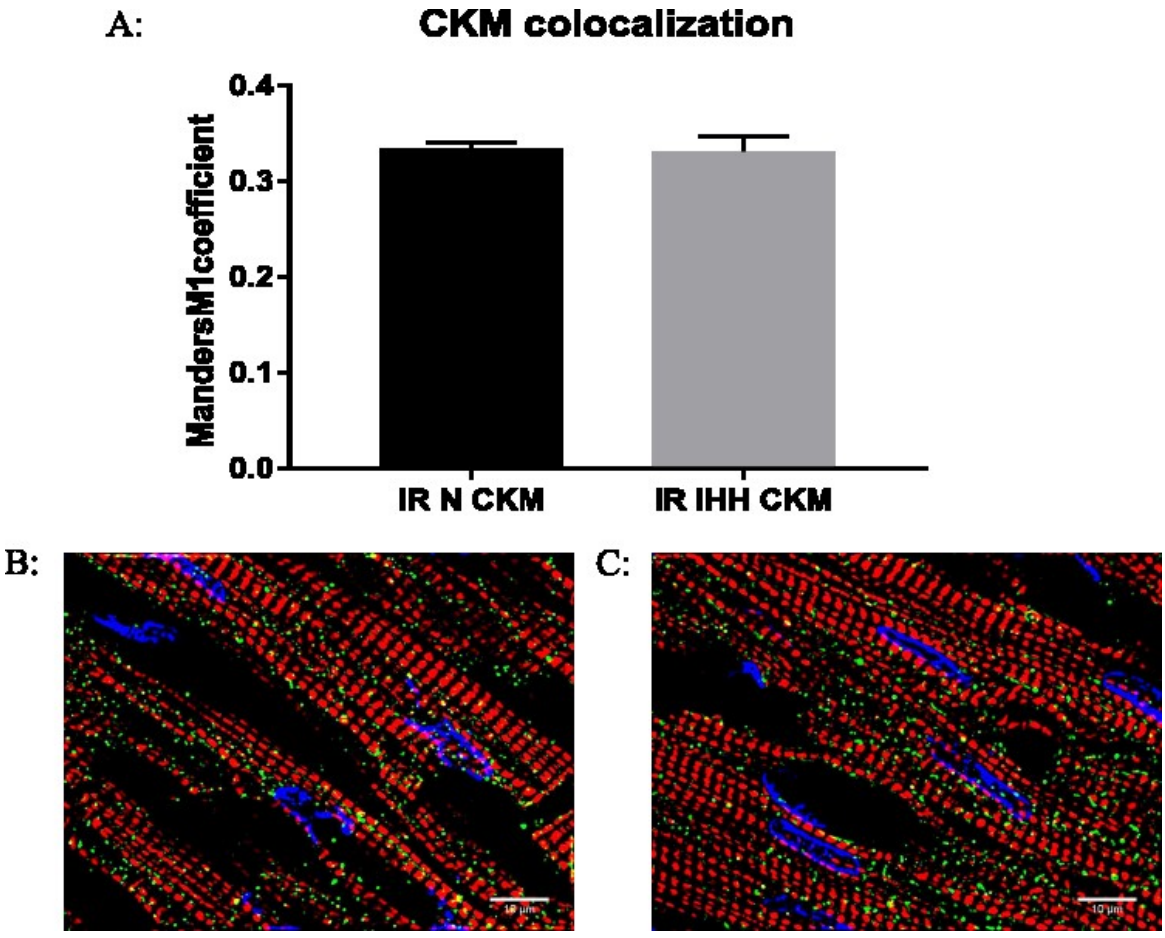

Fig. 13: *Colocalization of CKM with M lines represented by Manders' M1 coefficient (A). Values are mean ±SEM (n = 3). Representative images of longitudinal cryosections showing colocalization of CKM and actin with nucleus in the myocardium of LV after IR in normobaric rat (B) and rat adapted to IHH (C). Red color represents staining of actin by*

phalloidin, blue color represents nuclear DAPI staining and green color represents the specific CKM staining.

Localization of CKB in M lines after IR injury was significantly lower in IHH group when compared to controls. Strong negative correlation of CKB and M line marker in IHH group (M1 = 0.09467, n = 3) represents 64 % lower Manders M1 coefficient than weak negative correlation of control animals (M1 = 0.257, n = 3).

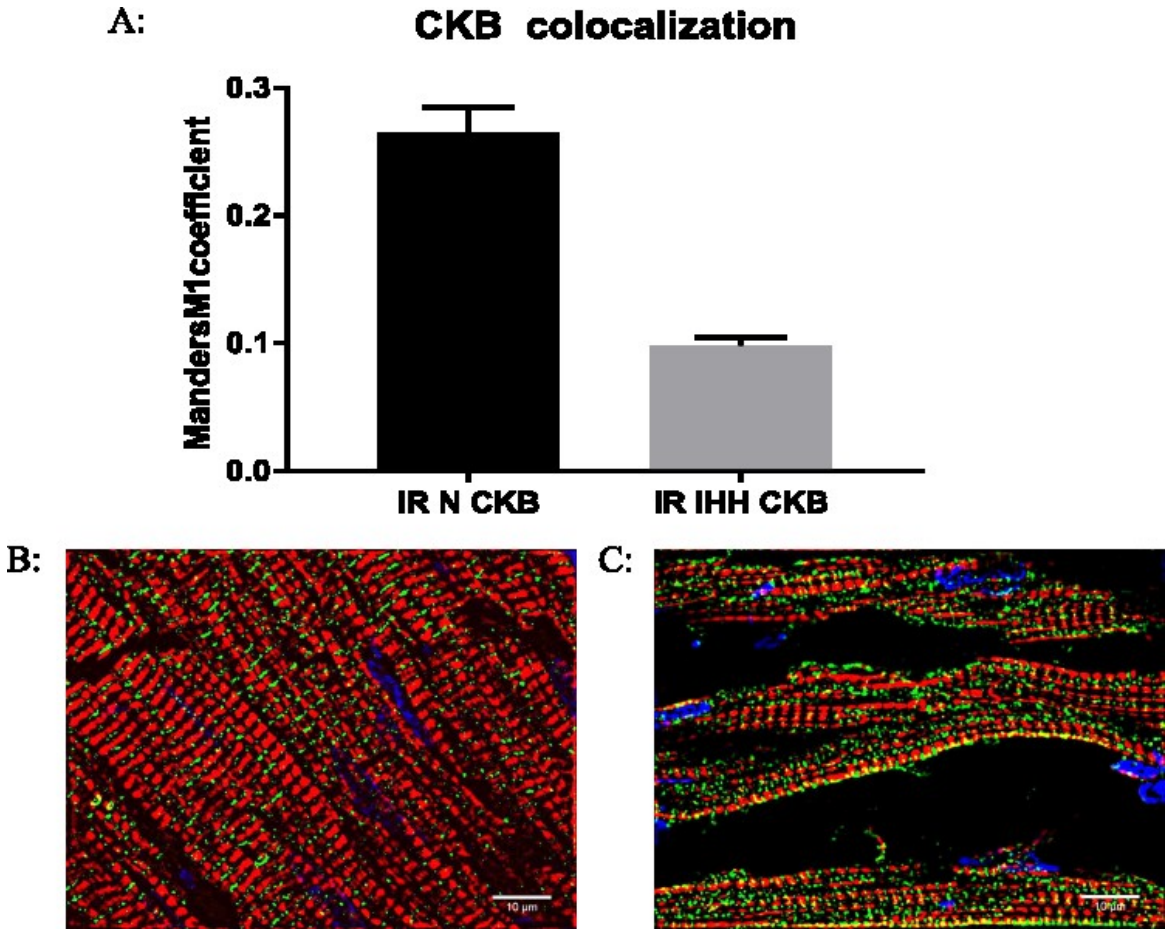

Fig. 14: *Colocalization of CKB with M lines represented by Manders' M1 coefficient (A). Values are mean ±SEM (n = 4). Representative images of longitudinal cryosections showing colocalization of CKB and actin with nucleus in LV myocardium after IR in normobaric rat (B) and rat adapted to IHH (C). Red color represents staining of actin by*

phalloidin, blue color represents nuclear DAPI staining and green color represents the specific CKB staining.

5.3.4. CK colocalization after ischemia and IR

Comparison of CK isoenzymes colocalization in control, ischemic and IR groups in both adaptations revealed different trends between adaptations. IHH did not follow the increase of CKM colocalization after ischemia observed in normobaric group. Manders M1 coefficient was decreased by 65 % (M1 of IHH control 0.2566 and M1 of IHH ischemic group 0.08635). Accordingly to ischemia, trend in IR possessed the opposite manner – e.g. in normobaric animals the colocalization decreased to the level similar as in control group, while IHH animals showed significant increase in CKM colocalization with the M line after IR. Manders M1 coefficient for CKM - M line colocalization increased by 33 % (M1 of normobaric control 0.3438, M1 of normobaric ischemic group 0.5413).

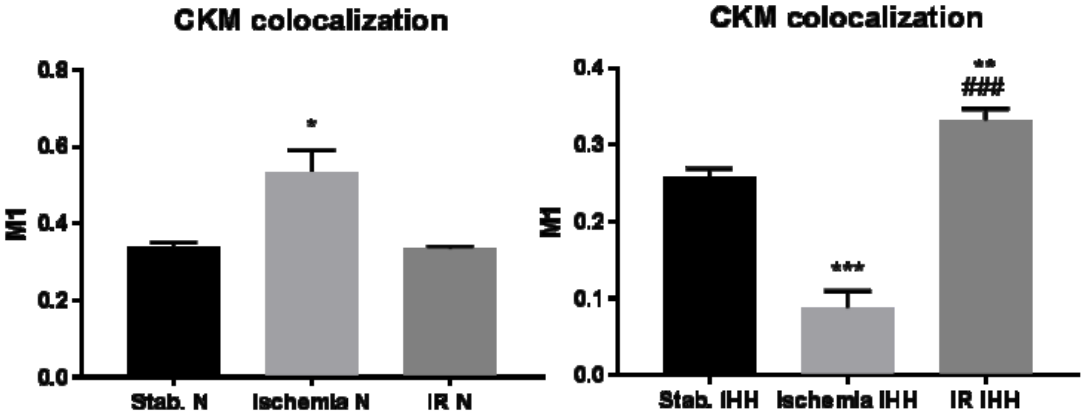

Fig. 15: *Colocalization of CKM with M lines represented by Manders' M1 coefficient. Values are mean ±SEM (n = 3, 4, 2 respectively for each paired adaptation) P < 0.05 * vs. stab. # vs. ischemia*

Comparison of CKB colocalization with the M line between normobaric control group and ischemic group revealed significant increase (254 % increase, M1 of normobaric control 0.1325, M1 of normobaric ischemic group 0.5159), which was not observed in IHH

animals. IHH animals did not show a significant difference between CKB and the M line colocalization after ischemia, but colocalization decreased after IR for both adaptations, although only for IHH animals colocalization coefficient dropped below the value of the control group (M1 of IHH control 0.178 and M1 of IHH IR 0.09467) while value of the coefficient representing the CKB - M line colocalization for normobaric myocardium after the IR was one fold higher than value for normobaric control group (M1 of normobaric control 0.1325 and M1 of normobaric IR 0.257).

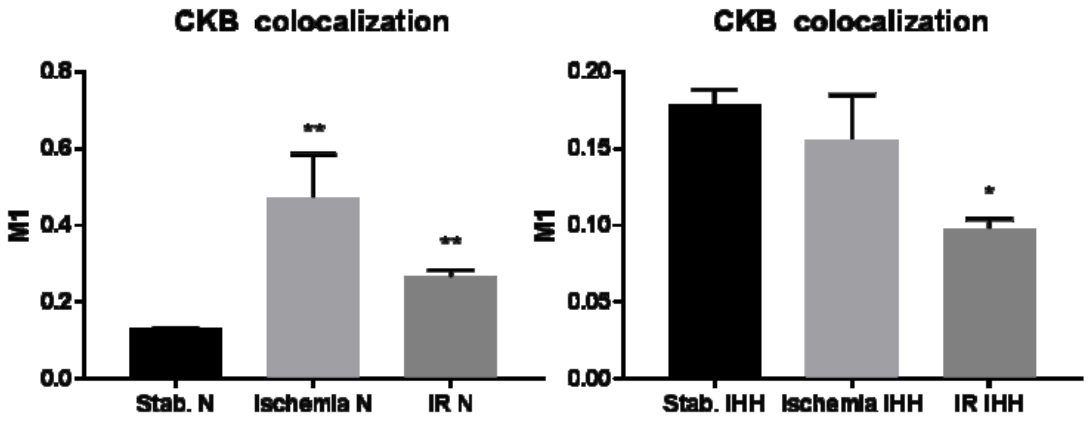

Fig. 16: *Colocalization of CKB with M lines represented by Manders' M1 coefficient. Values are mean ±SEM (n = 3, 4, 2 respectively for each paired adaptation) P < 0.0. * vs. both.*

6. Discussion

Unsustainable overload and ischemic insult lead to a HF, an abundant cause of death in many developed countries. Mammalian heart developed a complex machinery of adaptive mechanisms to battle overload and maintain steady and sufficient blood flow, energy homeostasis and oxygenation of the myocardium. The most critical adaptive mechanism is cardiac remodeling – hypertrophy and angiogenesis. Under physiological stress conditions, hypertrophied myocardium maintains cardiac function and angiogenesis support proper nourishment of cardiomyocytes, therefore hypertrophy in physiological range is classified as compensatory or adaptive. Prolonged stress signalization and/or pathological stimuli can amplify hypertrophy, leading to an abnormal growth of cardiomyocytes and ECM. Hypertrophy causing decreased cardiac function is classified as maladaptive and is associated with fibrosis, metabolic changes, inflammatory cytokines, disproportionate growth and even cellular dysfunction (Shimizu & Minamino, 2016).

Physiological and pathological phase of the hypertrophy are commonly accepted as two consecutive stages of cardiac remodeling and both type and the duration of the stimuli are determinants of hypertrophic response. To make transition between stages possible, different molecular pathways or regulation of the intensity of the signaling must be involved. The most distinct factor accompanying respective stages of cardiac hypertrophy is energy metabolism. Thus, energy metabolism holds a dominant place in cardiac hypertrophy, determining phenotype and susceptibility of myocardium in course of remodeling (Rimbaud et al., 2009; Shiojima et al., 2005).

Understanding of transition between physiological and pathological hypertrophy might offer a possible therapeutic approach for treating cardiovascular pathologies associated with cardiac hypertrophy. Regardless of whether hypertrophy is the reason or consequence of pathology, the decision for future pharmacological and medical studies regarding cardiac hypertrophy is between outright attenuation of hypertrophy and prevention of transition to detrimental phase. To answer this question, it is important to examine underlying mechanism and possibly beneficial adaptations of physiological hypertrophy, mainly those involved in energetic homeostasis.

Prenatal heart is exposed to different environmental conditions than developed adult heart and this difference is also visible in CK isoenzymes expression. CKBB with its higher efficacy in mild hypoxic condition is the major cytosolic isoform of developing heart. Interesting transitory step during cardiomyocyte differentiation was observed in vivo, when CKMB heterodimer replaces CKBB before CKMM becomes major isoform of terminally differentiated cardiomyocyte (Fischer et al., 2010). This prenatal adaptation to low pO_2 condition might be mimicked by heart in similar state during development of cardiac hypertrophy.

In the present study, we have found that compensatory state of hypertrophied heart of 4 months old SHR significantly increased association of CKM within the M lines. This supports the hypothesis, that strengthening of the CK system plays an important role in the enhancement of cardiac function during physiological hypertrophy. Association of CKB with the M line was not affected, suggesting that CKM isoform is sufficient in maintaining CK shuttle under physiological adaptive responses to hypertrophy.

However, in 13 months old SHR, in which the hypertrophy possesses pathological significance, the colocalization of CKM - M line returned to the control lever of WKY and this was partially compensated by significant increase of CKB in the M line. Overloaded myocardium which is chronically exposed to high energy and oxygen turnover together with decreased oxygen availability and decline of pH value due to hypertrophy provides conditions favorable for the increased expression and activity of CKB isoform.

Increased CKB association and lower CKM association with M lines in older animals confirms our expectations of CKB isoform replacing CKM in more severe hypertrophy. This result fits in observed phenomena of transition to expression mimicking fetal gene program (Hoerter et al., 1991; Pauletto et al., 1989) and confirms that increased CKB protein is indeed associated with the M lines, participating in creatine shuttle between mitochondria/glycolysis sites and myosin ATPases. Whether it is a CKBB or possibly CKMB dimer remains unclear and will be a subject of our subsequent study, based on results of this thesis.

Obtained results show clear distinction between metabolic isoenzymes localization in two stages of hypertrophy. Therefore, control over transition from adaptive to maladaptive hypertrophy might lay in expression and localization of metabolic systems components. Present results show obvious tendency of dynamics between CKM and CKB isoforms association with the M lines in course of hypertrophy and subsequent study will observe this trend in older animals with developed HF symptoms (Conrad et al., 1995) and complement observation with control WKY animals of relevant age to exclude role of the ageing. Consecutive study will also direct possibility, that the reason for CKM dissociation from the M lines is EH-myomesin (Schoenauer et al., 2011) overexpression and loss of CKM binding site.

In the second part of our study we focused on HK isoenzymes and dynamics in subcellular localization of HKs during development of the hypertrophy. Experiments focused on HK system were based on observed essential role of HK in attenuation of cardiac hypertrophy (McCommis et al., 2013). Developing cardiac hypertrophy is associated with increase in ROS generation, metabolic plasticity and, in the late stage, mitochondrial dysfunction (Jüllig et al., 2008; Takimoto & Kass, 2007). We hypothesized that HKs might provide crucial helping hand to delay onset of the mitochondrial dysfunction and to keep mitochondrial integrity and elevated oxidative metabolism by association with the outer mitochondrial membrane. HK1 – OXPHOS association increased significantly for both younger and older SHR rats in comparison with control 4 months old WKY rats, but HK2 and HK3 – OXPHOS association did not change in hypertrophied myocardium of 4 months old animals. This result confirms our hypothesis and reveals that HK1 isoform supports mitochondrial respiration in physiological hypertrophy by increased ADP supply to ATP synthase.

Decreased association of HK2 and HK3 with VDAC observed in older SHR group might represent an early onset of the mitochondrial dysfunction. With extensively described decrease in fatty acid oxidation, glucose utilization serves as a compensatory mechanism (Dávila-Román et al., 2002). Observed decreased colocalization of HK2 and HK3 with OXHOPS corresponds to overall attenuation of the metabolism in developed cardiac hypertrophy. Therefore, we propose that dissociation of HK2 and HK3 from outer

mitochondrial membrane is possibly mediated by the decay of oxidative glucose metabolism or glycogen depletion which might play an important role in dysregulation of energy metabolism in cardiomyocytes. This result is in accordance with already observed attenuation of the cardiac hypertrophy by increased HK2 expression (McCommis et al., 2013). HK2 is in literature described as a cardioprotective isoform (Waskova-Arnostova et al., 2015) and dissociation of HK2 from mitochondrial membrane in developed hypertrophy might be related to the observed lower tolerance of SHR strain to IR injury (Bélichard et al., 1988). Similarly, dissociation of HK3 in hypertrophied hearts might contribute to destabilization of mitochondrial potential during IR (John et al., 2011; Wyatt et al., 2010).

Third part of the study focused on dynamics in subcellular localization of CK isoenzymes after ischemia and IR in the heart of the rats adapted to IHH. Association of CK with M lines showed significant differences between control animals kept in normobaric conditions and animals adapted to hypoxia. Higher tolerance of IHH adapted animals to IR was already well described (Kolář, 2004). We hypothesized that CK in rats adapted to hypoxic conditions plays a role in this protective effect. Association of CKM with the M lines in severely hypertrophied hearts of IHH rats is comparable to that observed in older SHR. Steep increase in CKB association with the M line in normobaric group after ischemia is consistent with the hypothesis of activation of fetal genes expression in hypoxia and might present an adaptive response of myocardium to lowered pO_2. However, adaptive response in attempt to sustain contractility might not prove to be protective, as it leads to the ATP/ADP depletion.

On the other hand, no significant response in CKB – M lines colocalization after ischemia was observed in hypoxic group and both M1 coefficients of CKM and CKB colocalization with M lines were significantly lower in comparison with normobaric group. Impairment of CK system between mitochondria and myosin ATPases and thus decreased contractility leads to decrease in ATP consumption. Lower contractility protects heart from energetic imbalance threatening the heart during ischemia.

Another opposite tendency was observed in significant increase of CKM association with M lines after IR in hypoxic animals. Strong association of CKM with myosin ATPases promotes better contractility and reestablishment of normal cardiac function after ischemia. This result differentiates significantly from control animals and offers an explanation of cardioprotective effect described for IHH animals. In case of CKB isoenzyme, results show decrease in association with M lines after IR. With strengthened CKM there is no need for compensation by CKB and tight structure of M line might not provide enough space for additional CK.

7. Conclusion

To summarize the knowledge about CK and HK systems this thesis described changes in isoenzymes subcellular compartmentalization in different developmental stages of cardiac hypertrophy, induced by hypertension or IHH. Furthermore, we observed these changes after ischemia and IR in IHH rats. Description of metabolic pathways involved in adaptive hypertrophy has important implications in clinical research, providing basic understanding of therapeutic targets to attenuate transition from physiological to pathological hypertrophy or a hypertrophic process at all. Difference in the expression of CK and HK isoforms during hypertrophy was already well described as well as effect of HK2 overexpression (McCommis et al., 2013; Ye et al., 2001).

Experiments conducted in this thesis confirmed our hypothesis that transition from adaptive to maladaptive stage of cardiac hypertrophy is accompanied by a substitution of CKM by the CKB in the M line of cardiomyocytes. Strengthened CKM colocalization in mild hypertrophy decreases with progression of hypertrophic growth and is substituted by increasing CKB association with M lines, which was not observed in mild hypertrophy. This result suggests adaptive, maybe even protective (concluding from IHH experiments and higher efficacy in low pO_2 conditions) role of CKB isoenzyme in development of hypertrophy induced by hypertension. CKM - M lines colocalization in IHH rats was similar to that of older SHR, confirming dissociation of CKM from the M lines in another model of severe hypertrophy. Progressive dissociation of CKM after ischemia might decrease contractility and therefore serve as a protective mechanism against ATP depletion. Role of CKM in cardioprotective effect of IHH reaches its potential after IR, when rapid association of CKM with M lines restores contractility. This increased tolerance to IR is similar to cardioprotective effect of cold adaptation. In contrast with hypertrophy induced by hypertension, no compensation of creatine shuttle by CKB isoenzyme was observed, as CKM adaptation seems sufficient.

Increased association of HK1 with mitochondria compensates faltering metabolism of hypertrophied heart in both physiological and pathological hypertrophy. Dissociation of cardioprotective HK2 and HK3 from mitochondria after transition to the pathological

hypertrophy contributes to the increased probability of mitochondrial PTP opening ant consecutive destabilization of mitochondrial potential.

Acquired results suggest following: 1. During development of cardiac hypertrophy, not only expression shifts towards adaptive fetal program, but also colocalization in the M lines transits towards CKB isoenzyme, even though no known binding partner for CKB was described in the M line 2. Association of HK with mitochondria is consistent with decreasing contractile function 3. Differences in CK isoenzymes dynamic association with the M line might play an important role in cardioprotective effect of IHH adaptation. To the best of our knowledge, this is the first study relating these aims.

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